Platelet function in subjects with diabetes mellitus and hyperlipidaemia.

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PLATELET FUNCTION IN SUBJECTS WITH DIABETES MELLITUS AND HYPERLIPIDAEMIA

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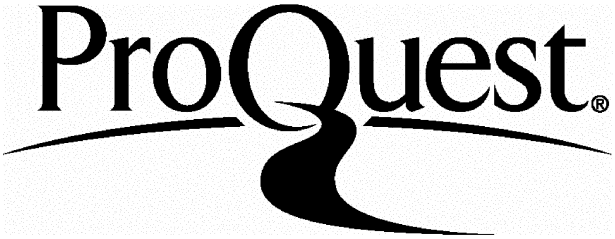

SUMMARY

Premature vascular disease is an important cause of morbidity and mortality in Western countries and two groups particularly at risk from premature and extensive vascular disease are diabetic and hyperlipidaemic patients. In addition diabetics develop a specific microvascular disease affecting the capillaries resulting in retinopathy, nephropathy and contributing to neuropathy.

Many factors may be involved in the pathogenesis of vascular disease in these two patient groups. In this thesis particular attention has been given to the detection of enhanced platelet function as platelets are important in thrombosis which is often the final event leading to the occlusion of an atheromatous artery and platelets may contribute to the development of vascular disease.

The results reported in this thesis suggest that in diabetic and hyperlipidaemic patients there is evidence of increased *in vivo* platelet release reaction as evidenced by the finding of elevated levels of the platelet-specific proteins, β-thromboglobulin and platelet factor 4 in platelet-poor plasma from these patients. There is evidence also of enhanced activity of the platelet prostaglandin pathway in response to stimulation with arachidonic acid in these patients. These abnormalities did not appear to be related to pre-existing vascular disease in the patients studied. Furthermore platelets from diabetic subjects show diminished sensitivity to the anti-aggregatory effects of prostacyclin and enhanced platelet aggregation to the aggregatory agonists collagen and arachidonic acid when studied in whole blood. In addition the experimental diabetic rat produces less aortic prostacyclin than control rats.

These results provide evidence of enhanced platelet function in patients with diabetes and hyperlipidaemia which may contribute to their increased vascular risk. However these conclusions can only remain tentative until it is possible to perform long-term prospective studies in these patients.

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ABBREVIATIONS

HDL	-	high density lipoprotein
LDL	-	low density lipoprotein
VLDL	-	very low density lipoprotein
IDL	-	intermediate density lipoprotein
g	-	acceleration due to gravity
pH	-	minus log of hydrogen ion activity
w/v	-	weight by volume
%	-	percentage
≃	-	approximately
kg	-	kilogram
g	-	gram
mg	-	milligram 10^{-3} gram
µg	-	microgram 10^{-6} gram
ng	-	nanogram 10^{-9}gram
mol	-	mole
mmol	-	millimole 10^{-3} mole
µmol	-	micromole 10^{-6} mole
LCAT	-	lecithin-cholesterol acyltransferase
l	-	litre
ml	-	millilitre 10^{-3} litre
µl	-	microlitre 10^{-6} litre
mm	-	millimetre 10^{-3} metre
µm	-	micrometre 10^{-6} metre
nm	-	nanometre 10^{-9} metre
^{o}C	-	degrees centrigrade
mU	-	milli unit
SDS	-	sodium dodecyl sulphate
sec	-	second
min	-	minute
h	-	hour
ATP	-	adenosine triphosphate
ADP	-	adenosine diphosphate
AMP	-	adenosine monophosphate

/ contd...

ABBREVIATIONS (contd.)

PRP	-	platelet-rich plasma
PPP	-	platelet-poor plasma
Ω	-	ohm
SD	-	standard deviation
SEM	-	standard error of the mean
r	-	correlation coefficient
R	-	Spearman correlation
p	-	probability
NS	-	not significant

INTRODUCTION

Atherosclerosis is the commonest cause of death in Western populations. Mortality figures from the Registrar General's returns for England and Wales for 1974 indicate that ischaemic heart disease is the largest single cause of death in middle-aged men and a major cause of death in middle-aged women (Registrar General, 1977).

By the time the clinical consequences of atherosclerosis develop, the disease is often in an advanced stage. In this situation, where arteries are constricted with irregular lumens lined by calcified necrotic debris, therapy can often be no more than palliative. Ideally, therefore, attention should be concentrated on prevention.

It is probable that different stimuli can damage the arterial wall and produce the same final pathology of atherosclerosis. Attention has been focussed on factors identified from epidemiological studies as being associated with increased risk of development of clinical evidence of atherosclerosis. The term 'risk factor' is widely used to describe these features although not necessarily indicating a causal relationship.

A number of factors including hypertension, cigarette smoking, obesity, physical inactivity and stress have been implicated in the accelerated development of coronary artery disease (Joint Working Party of the Royal College of Physicians and the British Cardiac Society, 1976). It is not proposed to discuss these factors individually and here attention will be focussed on two groups particularly at risk from premature vascular disease, namely the diabetic and hyperlipoproteinaemic populations.

This thesis is concerned with the assessment of platelet function in these two high risk populations as enhanced platelet function may contribute to their increased vascular risk. In this introduction I will therefore outline the problem of diabetic vascular disease and describe the hyperlipoproteinaemias and their relationship to vascular disease.

Diabetes mellitus is an important cause of secondary lipid and lipoprotein abnormalities and I will describe the altered lipoprotein metabolism in diabetes as the results of the platelet studies to be reported will be correlated not only to glycaemic control but also to individual lipid and lipoprotein concentrations in the diabetic subjects. I will describe platelet physiology and highlight the recent understanding of the importance of the prostaglandin pathway in platelets. The importance of platelet/vessel wall interactions has been emphasized in recent years and I will detail the physiology of prostacyclin which probably is important in this regard. Platelets will be discussed in relation to thrombosis and atherogenesis and the rationale for studies of platelet function in diabetic and hyperlipoproteinaemic subjects will be discussed.

Diabetes and Vascular Disease

Large vessel disease has become the commonest cause of death in the diabetic population. Although the average life span of diabetics has greatly increased since the advent of insulin, they still have a shorter life expectancy than non-diabetics. The already high incidence of vascular disease in non-diabetic Western populations appears to be still higher in diabetics; cardiovascular disease was responsible for 79% of deaths in diabetic patients at the Joslin Clinic in 1966, contrasting with a 54% death rate due to cardiovascular disease in the general population during the same year (Marks and Krall, 1971).

Although there have been suggestions of a specific diabetic large vessel disease (Lundbaek, 1973) there is little evidence to suggest that atherosclerotic lesions in diabetics are distinct from those in non-diabetics (Strandness *et al*, 1964). However, atherosclerosis appears to be more extensive and to develop at an earlier age in diabetic patients (Robertson and Strong, 1968). These pathological findings are in agreement with clinical studies, the most convincing evidence coming from the

prospective community study in Framingham. All major clinical manifestations of atherosclerosis were found to have an increased incidence in diabetics (Garcia, *at al*, 1974).

Many factors may contribute to the increased incidence of large vessel disease in diabetic patients as in non-diabetic subjects, however, this increased incidence in diabetes is not fully explainable in terms of known risk factors (Jarrett *et al*, 1982). Therefore other metabolic abnormalities have been sought in diabetic subjects which might contribute to the increased vascular risk. In this thesis platelet function will be studied in diabetic patients because of the postulated role of platelets in the early lesions of atheroma and their undoubted importance in the development of thrombus on pre-existing atheromatous plaques which leads to final arterial occlusion.

In addition to the high incidence of atherosclerosis, diabetics also develop a specific vascular disease involving small vessels. This micro-angiopathy, which is characterised by a thickened but paradoxically leaky capillary basement membrane, makes an important contribution to morbidity and mortality in the diabetic population principally through retinopathy, nephropathy and neuropathy (Keen and Jarrett, 1982).

It is generally agreed that the development of microvascular disease in diabetics is a result of the metabolic derangement plus time. However the actual pathogenesis of the capillary lesion is poorly understood. It has been postulated that abnormal platelet function may play a role in the development of microvascular disease (Colwell *et al*, 1978). Therefore in this thesis the diabetic patients studied will be carefully clinically examined to determine the presence or absence of the different diabetic vascular complications and the results of the parameters of platelet function will be analysed on this basis.

Lipoproteins

The major plasma lipids, triglyceride, cholesterol esters, free cholesterol and phospholipids are held in solution in the form of complex water-soluble molecular aggregates known as lipoproteins. Various analytical techniques, including electrophoresis, preparative ultra-centrifugation and measurement of lipid and protein contents of the various lipoprotein classes, have led to a dramatic increase in the understanding of the composition of these complex particles and have provided the basis for their classification (Table 1)

Chylomicrons are the largest of the lipoprotein species, and are normally found in plasma only after a meal. They serve to transport dietary fats from the small intestine via the lymphatic system to the thoracic duct where they enter the blood stream. They are rich in triglyceride and contain only 1-2% protein by weight. The apoprotein component of newly formed chylomicrons is apoprotein B-48 and in lymphatics and circulation chylomicrons acquire other apoproteins of the C and E groups (Havel *et al*, 1973). The triglyceride content of chylomicrons is hydrolysed to free fatty acids by the enzyme lipoprotein lipase which has its site of action on capillary endothelium of skeletal muscle and adipose tissue (Blanchette-Mackie and Scow, 1971). The free fatty acids are either oxidised for fuel or re-esterified in adipose tissue for storage as triglyceride. The chylomicron remnant after removal of the triglyceride core dissociates from the capillary endothelium and is taken up by liver via a receptor-mediated process involving apoprotein E.

Very low density lipoprotein (VLDL) serve to transport endogenously synthesized triglyceride. They are synthesized in the liver and to a lesser extent the intestine (Roheim *et al*, 1966; Windmueller *et al*, 1973; Noel and Rubinstein, 1974). VLDL secreted by the perfused liver contain both apoprotein B and apoprotein C whereas apoprotein C is absent from VLDL

Table 1a. Chemical Composition of Serum Lipoproteins

	Protein	Triglyceride (% by weight)	Cholesterol	Phospholipid
Chylomicrons	2.5	85	4	8
VLDL	10	50-55	15	18
LDL	20-25	12	30	22
HDL	50	6	15	25

Table 1b. Physical Properties of Serum Lipoproteins

	Diameter (nm)	Molecular Weight (daltons)	Hydrated Density (g/mℓ)	Electrophoretic mobility
Chylomicrons	80.0	10^9	0.93	origin
VLDL	25.0-80.0	10^7	0.97	pre-beta
LDL	17.5-25.0	2.3×10^6	1.03	beta
HDL_2	8.5-15.0	3.6×10^5	1.09	$alpha_2$
HDL_3	7.0-9.0	1.7×10^5	1.15	$alpha_3$

Table 1c. Apolipoproteins of Human Serum

APOLIPOPROTEIN	DENSITY CLASS	MOLECULAR WEIGHT (daltons x 10^{-3})	CONCENTRATION (mg/ℓ)
A-1	HDL	28	800-1200
A-11	HDL	17	300-500
A-111	HDL	21	20-40
B	LDL, VLDL	275	700-900
C-1	VLDL, HDL	7	30-70
C-11	VLDL, HDL	8.5	30-50
C-111	VLDL, HDL	8.5	80-120
E	VLDL, HDL	39	30-60
D-2	HDL	7	10-20

(Assmann and Schmitz, 1979)

in intestinal lymph (Windmueller *et al*, 1973). It is not known whether hepatic VLDL are secreted with a full component of apoprotein C, but available evidence suggests that apoprotein C is transferred from high density lipoprotein (HDL) in the circulation (Herbert *et al*, 1978). VLDL, like chylomicrons, undergo hydrolysis of their triglyceride content by the enzyme lipoprotein lipase acting in peripheral tissues. During the catabolism of VLDL the majority of apoprotein B is conserved while surface components including phospholipid and apoprotein C transfer to HDL. Turnover studies using radioactively-labelled lipoproteins have demonstrated that VLDL are mainly converted to low density lipoprotein (LDL) in the circulation via intermediate density lipoproteins (IDL)(Eisenberg and Levy, 1975). IDL can also be taken up directly by the liver.

LDL are cholesterol-rich particles responsible for transport of approximately 70% of the total plasma cholesterol. These particles are not normally synthesized by the liver but are virtually all derived from the metabolism of VLDL (Eisenberg and Levy, 1975). LDL serve to deliver cholesterol to peripheral cells and the pathway of catabolism of LDL has been delineated by Brown and Goldstein (1976). Briefly, there are specific high affinity receptors on cells which recognise LDL apoprotein B. The receptor bound LDL is internalised by a process of absorptive endocytosis. The resultant endocytotic vesicle fuses with lysosomes where the cholesterol esters of LDL are hydrolysed to yield free cholesterol. The free cholesterol diffuses from the lysosomes into the cell cytoplasm where it may be used for membrane synthesis. In addition this cholesterol has regulatory actions on cholesterol metabolism within the cell; it suppresses synthesis of LDL receptors, suppresses cholesterol synthesis and stimulates its own re-esterification. This receptor mediated pathway accounts for about two-thirds of the LDL which is degraded daily ≃ 1000 mg LDL-cholesterol. In addition a further 500 mg of LDL-cholesterol is cleared from plasma by a receptor-independent scavenger mechanism.

HDL particles account for about 20-30% of the total plasma cholesterol. There is considerable heterogeneity within HDL and the particles contained within the HDL fraction arise from several different sources including direct synthesis by the liver and intestine and as a bi-product of the metabolism of triglyceride-rich lipoproteins. Whilst the liver is able to synthesize both apoprotein A and apoprotein C, HDL of intestinal origin appears to contain apoprotein A only (Windmueller *et al*, 1973). HDL may facilitate net cholesterol removal from all membranes *in vivo* and serve to transfer cholesterol from peripheral cells to the liver for excretion. HDL cholesterol is the preferred substrate for lecithin-cholesterol acyl-transferase (LCAT) (Akanuma and Glomset, 1968) which catalyzes the esterification of cholesterol by transfer of fatty acid from the C-2 position of phosphatidyl-choline. The reaction takes place mainly on or within the lower molecular weight sub-fractions of HDL (Glomset *et al*, 1966). An important activator of this enzyme is apoprotein A-1, the main protein component of HDL (Fielding *et al*, 1972). The HDL/LCAT enzyme complex can therefore act as an acceptor of cellular cholesterol.

Lipoproteins and Vascular Disease

One of the most striking features of the atherosclerotic lesion is the accumulation of cholesterol ester which deposits in the proliferating smooth muscle cells and in the surrounding interstitial space of the arterial intima and media. Although the histology of the atheromatous plaque is complex, a number of lines of evidence suggest that cholesterol may play a central role in its development. Severe atherosclerosis can be produced in many species of experimental animal by feeding a diet that raises the plasma cholesterol level (Roberts and Straus, 1965). Proliferative thickening of the arterial wall can be produced in animals by trauma, such as hypertension or by destruction of the endothelium by physical or chemical methods. However the lesion formed remains a simple scar unless the

animal is made hypercholesterolaemic, when severe atherosclerosis develops (Wissler, 1973).

Studies in human populations have shown that symtomatic atherosclerosis does not frequently develop even when there are predisposing factors, such as cigarette smoking, hypertension or diabetes mellitus, unless the mean concentration of serum cholesterol in the population exceeds 4 mmol/l (Epstein, 1971; Stamler, 1973 and Keys, 1975). A single gene-determined disorder familial hypercholesterolaemia, in which there are markedly elevated plasma and tissue cholesterol levels is accompanied by accelerated atherosclerosis at an early age without other contributing factors, such as hypertension, smoking or diabetes mellitus being present (Frederickson *et al*, 1978).

This apparent central role of cholesterol in the pathogenesis of the atheromatous plaque provoked many epidemiological and clinical studies which examined the relationship between plasma cholesterol and atherosclerosis. It is now long established that there is in fact a strong positive correlation between plasma cholesterol and atherosclerosis but stronger relationships are seen for the major cholesterol carrying lipoproteins - LDL and HDL. Increasing concentrations of plasma LDL cholesterol are strongly and independently predictive of coronary artery disease and account for the association seen with total cholesterol (Gordon *et al*, 1981). Equally HDL cholesterol concentrations are strongly and independently predictive of coronary disease and in some studies more so than LDL (Miller *et al*, 1977; Goldbourt and Medalie, 1979). However the association with HDL is inverse, in other words decreasing HDL cholesterol concentrations are associated with increasing risk of coronary artery disease.

Although these epidemiological findings have not yet been fully explained in biological terms more and more evidence is accumulating

implicating LDL and HDL in the biochemistry of atherosclerosis.

Cholesterol deposited in the atheromatous lesion is derived from plasma (Zilversmit, 1968) and LDL enters the arterial wall at a rate directly related to its plasma concentration (Nicoll *et al*, 1981). The foam cells so characteristic of atheroma may have their origin in macrophage monocytes which have receptors for chemically altered LDL. Unlike normal LDL receptors they are not down regulated by increasing cellular cholesterol concentrations (Goldstein *et al*, 1979). One type of chemically altered LDL taken up by macrophages resulting in massive cholesterol accumulation in these cells is malondialdehyde-modified LDL (Forgelman *et al*, 1980). Another mechanism by which LDL cholesterol might accumulate in cells is by fluid phase endocytosis which is independent of receptors (Steinberg, 1981).

LDL has other potentially important effects in relation to the pathogenesis of atherosclerosis. It can be directly toxic to arterial endothelial cells (Ross and Harker, 1976) and endothelial injury results in increased passage of LDL from plasma into the arterial intima. In addition LDL can be directly mitogenic to smooth muscle cells (Fless *et al*, 1982). As well as these potentially important pathogenic effects LDL has important interactions with blood platelets as will be discussed later.

It has been postulated that HDL might exert its apparent protective role against the development of atherosclerosis by returning cholesterol from peripheral cells to the liver for excretion (Miller and Miller, 1975). HDL with the enzyme LCAT (HDL cholesterol is the preferred substrate for LCAT and apoprotein AI, the main apoprotein of HDL is an important activator of the enzyme) might facilitate net cholesterol removal from cell membranes by acting as an acceptor for cellular cholesterol (Glomset, 1968). *In vitro* studies where HDL has been shown to enhance cholesterol efflux from cultured cells support this hypothesis (Stein and Stein, 1973; Stein *et al*, 1975). It is the HDL

subfraction HDL_3 which is the initial acceptor of cholesterol (Rothblat and Phillips, 1982) and one proposed pathway among others for return of cholesterol to the liver involves the conversion of HDL_3 to HDL_2 which in turn acquires apoprotein E to form HDL_c. HDL_c can be rapidly taken up by hepatic cells by specific receptor mediated processes which recognise apoprotein E (Mahley, 1982). Alternatively HDL may be produced in the periphery at a rate depending on cholesterol efflux from cells. This suggestion is based on the finding that cholesterol efflux from some cells is accompanied by secretion of apoprotein E (Basu *et al*, 1981). In addition to its possible role in reverse cholesterol transport HDL has other effects which may be important in the development of atherosclerosis. HDL_c which contains apoprotein E can compete with LDL for binding to the LDL receptor therefore reducing LDL uptake (Miller *et al*, 1977).

The relationship of increasing plasma triglyceride levels to vascular risk is not so well defined as for plasma cholesterol and the cholesterol-rich lipoproteins. In some prospective studies, an independent relationship between plasma triglyceride levels and the development of vascular disease has been reported (Carlson and Böttiger, 1972), but this relationship has not been a universal finding (Wilhelmsen *et al*, 1973; Rosenman *et al*, 1976). In a more recent prospective study (Pelkonen *et al*, 1977) of a large group of middle-aged Finnish men followed over a 7-year period, an independent association was found between cardiovascular mortality and fasting serum triglyceride levels. This study is open to criticism for a number of reasons. There was a substantial loss of the original cohort to follow-up, and in addition hypertension, an important predictor of vascular risk, was not included in the analysis. However, perhaps the most important criticism is that HDL levels were not measured. There is an inverse correlation between VLDL, the major triglyceride-rich lipoprotein and HDL and it is possible that the association between VLDL

and vascular risk is not independent and may be explained by the inverse relationship between VLDL and HDL (Hulley *et al*, 1980).

Chylomicrons do not appear to be related to atherosclerosis as in Type 1 hyperlipoproteinaemia where there is massive accumulation of these particles there does not appear to be an increased incidence of vascular disease. On the other hand recent evidence from *in vitro* studies does suggest that VLDL may play a role in the development of atherosclerosis through their ability to deposit lipid in cells of the arterial wall. Although normally VLDL are not recognised by the classical LDL receptor it appears that VLDL from hypertriglyceridaemic subjects can bind to and be taken up by the LDL receptor mechanism in cultured fibroblasts and this process is related to the apoprotein E content of the particles (Gianturco *et al*, 1983). In addition VLDL can be taken up by receptors on cultured macrophages which appear to be distinct from the LDL receptor and the receptor for modified LDL. This receptor has been designated the β-VLDL receptor (Kraemer *et al*, 1983). By this pathway therefore VLDL could produce massive lipid accumulation in macrophages.

Hyperlipoproteinaemias

Classification of the hyperlipoproteinaemias in the absence of an understanding of primary aetiological and pathophysiological mechanisms are mainly operational and have become possible with the development of techniques for the qualitative and quantitative separation of the individual lipoprotein species. The most widely accepted classification is based on measurement of fasting total serum cholesterol and triglyceride levels together with lipoprotein electrophoresis (Frederickson *et al*, 1967; Beaumont *et al*, 1970). In addition, ultracentrifugation of serum is important for the demonstration of the abnormal apoprotein B-containing lipoprotein of type III hyperlipoproteinaemia which floats in the $d < 1.006$ g/ml fraction. The new precipitation techniques allow for simple

measurement of LDL (Burstein *et al*, 1970) and VLDL (Ononogbu and Lewis, 1976) in patients with mixed elevations of cholesterol and triglyceride.

On the basis of these measurements, six lipoprotein phenotypes have been described (Table 2). This classification has limitations because it is based entirely on laboratory operational techniques. Firstly it is clear that a particular lipoprotein abnormality may result from more than one pathological mechanism; for example, the type IV abnormality may be an expression of decreased clearance of VLDL or due to over synthesis of VLDL or both. A second major limitation of the typing system is in the area of genetics. No specific type of hyperlipoproteinaemia can be considered to be genotypic and this limitation has been particularly emphasized by the description of familial combined hyperlipoproteinaemia which appears to be due to a single autosomal dominant gene although the plasma lipoprotein pattern may be variable in affected individuals (Goldstein *et al*, 1973).

Fasting serum concentrations of cholesterol and triglyceride, together with lipoprotein typing using cellulose acetate electrophoresis have been measured in a large carefully documented, randomly selected group of men and women employed in a light industrial factory in North-East London (Lewis *et al*, 1974). Despite the fact that the upper cut-off points for serum cholesterol and triglyceride concentrations were intentionally set high (7.75 mmol/l for cholesterol and 2.0 mmol/l for triglyceride), the prevalence of hyperlipoproteinaemia in this healthy population of 276 subjects was high; 17% of men and 8% of women having hyperlipoproteinaemia. 4.3% of men and 4.8% of women, aged 40-69 years, had hypercholesterolaemia and 14% of men and 3% of women in this age range had hypertriglyceridaemia (Lewis *et al*, 1974). In another study (Lorimer *et al*, 1974) a similar high frequency of hyperlipoproteinaemia was found in a series of 4,385 healthy males living in Scotland. The overall prevalence of hyperlipo-

Table 2a. The Hyperlipoproteinaemias

TYPE	APPEARANCE OF SERUM	LIPIDS			LIPOPROTEIN PATTERN			ELECTROPHORETIC MOBILITY
		CHOLESTEROL	TRIGLYCERIDES	FASTING CHYLOMICRONS	LDL	VLDL	HDL	
I	'Cream layer'; Clear infranatant	Normal or ↑	Greatly ↑	Present	Normal or ↓	Mildly ↑ Normal or ↓	Absent	Chylomicron at origin
IIa	Clear	↑	Normal	Absent	↑	Normal or ↓	Normal or ↓	Beta band ↑
IIb	Clear or faintly turbid	↑	↑	Absent	↑	↑	Normal or ↓	Beta band ↑ pre-beta band ↑
III	usually turbid may be also faint 'cream layer'	↑	↑	Present may be ↑	d 1.006† -1.019↑ d 1.019-1.063↓	↑ *	Normal or↓	'Broad beta' band
IV	usually turbid	Normal or ↑	↑	Absent	Normal or ↓	↑	Normal or ↓	pre-beta band ↑
V	'Cream layer'; Turbid infranatant	Normal or ↑	Greatly ↑	↑	Normal ↓	↑	Usually ↓	Chylomicrons and pre-beta band ↑

† Intermediate density lipoproteins

* 'Floating' beta lipoproteins - density <1.006g/mℓ with β electrophoretic mobility

Adapted from Gotto and Jackson (1978)

Table 2b. Primary and Secondary Causes of Hyperlipoproteinaemia

TYPE	PRIMARY	SECONDARY
I	Familial lipoprotein lipase deficiency	Diabetes mellitus; hypothyroidism; macroglobulinaemia; multiple myeloma
II	Familial hypercholesterolaemia; Familial combined hyperlipoproteinaemia; Polygenic hypercholesterolaemia.	Hypothyroidism; nephrotic syndrome; dysgammaglobulinaemia; hepatic obstruction; hepatic disease; acute porphyria; androgenic steroids
III	Familial broad beta disease	Hypothyroidism; diabetes mellitus; systemic lupus erythematosus
IV	Familial hypertriglyceridaemia Familial combined hyperlipoproteinaemia	Alcoholism; diabetes mellitus; hypothyroidism; nephrotic syndrome; uraemia; oestrogens; pregnancy; corticosteroids; pancreatitis; glycogen storage disease; dysgammaglobulinaemias; systemic lupus erythematosus
V	Familial hypertriglyceridaemia; Familial mixed or type V hyperlipoproteinaemia; Familial combined hyperlipoproteinaemia	Alcoholism; diabetes mellitus; hypothyroidism; nephrotic syndrome; pancreatitis; glycogen storage disease; dysgammaglobulinaemias; systemic lupus erythematosus

(Gotto and Jackson, 1978; Glueck, 1977)

proteinaemia was 15.3%, 12% with type IV hyperlipoproteinaemia and 3% with type II hyperlipoproteinaemia.

Lipoproteins and Diabetes Mellitus

A large number of studies have demonstrated a high frequency of lipid and lipoprotein abnormalities in diabetic subjects. In a large group of newly diagnosed diabetics (both insulin dependent and insulin independent) over 40% had fasting plasma triglyceride concentrations greater than 2 mmol/l (Hayes, 1972). Similar results were reported in a large group of insulin dependent children prior to treatment (Chance *et al*, 1969). A high frequency of hypertriglyceridaemia is also found in diabetics established on treatment. In one study of 102 non-insulin dependent diabetics over 30% had fasting triglyceride concentrations above 2 mmol/l (Hayes, 1972) and of 98 randomly-selected diabetics of whom about a third were insulin-taking 22% had fasting triglyceride concentrations greater than 1.7 mmol/l (Wilson *et al*, 1970). The hypertriglyceridaemic group in this study were distinguished by greater hyperglycaemia and relative body weight. In a large mixed group of diabetic patients aged 5-85 years, diabetics over the age of 30 years had significantly higher triglycerides than non-diabetics but no differences were found in the under 30 age group (New *et al*, 1962). However fasting triglyceride levels in 270 juvenile diabetics on admission to Summer camps were higher than controls of comparable age (Kaufmann *et al*, 1975). Similarly an older group of diabetics studied as part of the Framingham community prospective study showed significantly elevated mean triglyceride levels in both male and female diabetics compared to non-diabetic subjects in the same community (Garcia *et al*, 1974).

It has been stated that "hypertriglyceridaemia is the hyperlipidaemia par excellence of the diabetic" (Albrink, 1974). However some studies have shown a high frequency of hypercholesterolaemia in diabetics. Cholesterol levels were significantly higher in 195 established diabetics aged 5-85 years

compared to controls throughout all age ranges (New *et al*, 1962). Similarly in a large group of young insulin dependent subjects mean cholesterol levels were higher than controls in both boys and girls (Kaufman *et al*, 1975). In the Framingham community prospective study mean cholesterol concentration was significantly higher in diabetic women but not in diabetic men (Garcia *et al*, 1974). Other studies have shown that the frequency of hypercholesterolaemia is similar to that of hypertriglyceridaemia in diabetics (Hayes, 1972; Wilson *et al*, 1970; Kaufmann *et al*, 1975; Schonfeld *et al*, 1974). More recently LDL-cholesterol concentrations have been measured in diabetic subjects. No difference in mean LDL-cholesterol concentrations was found in longstanding insulin dependent subjects compared to control subjects in a study from Helsinki (Nikkila and Hormila, 1978) but LDL cholesterol levels were higher in young insulin dependent subjects (Sosenko *et al*, 1980). Elevated LDL-cholesterol was found in insulin dependent men but not insulin dependent women in a study from London (Mattock *et al*, 1979) but no differences were seen for either sex in Tecumseh (Ostrander *et al*, 1980) or Framingham (Kannel *et al*, 1979). However among Pima Indians with non-insulin dependent diabetes LDL-cholesterol was significantly elevated (Howard *et al*, 1978). Although these findings with regard to LDL-cholesterol concentrations in diabetics are not consistent it is important to remember that LDL-cholesterol is a very powerful risk factor in all individuals including diabetics.

Since the re-awakening of interest in HDL and its apparent protective role in atherosclerosis several studies have been performed in diabetic populations. These studies have shown conflicting results but they are probably explained by differences in the diabetic groups studied. It is now becoming clear that HDL concentrations tend to be low in non-insulin dependent subjects (Howard *et al*, 1978; Lopes-Virella *et al*, 1977; Kennedy *et al*, 1978; Nikkila, 1978; Taylor *et al*, 1981). However in insulin dependent diabetics HDL-cholesterol concentrations tend to be equal to or

higher than in non-diabetic controls (Elkeles *et al*, 1978; Eckel *et al*, 1981; Wille and Aarseth, 1974). In a study from Helsinki the only difference between lipoprotein concentrations in longstanding insulin dependent diabetics and controls was that the former had significantly higher HDL cholesterol concentrations (Nikkila and Hormila, 1978). This elevation in HDL in insulin dependent diabetics appears to be due to an increase in the HDL_2 sub-fraction (Eckel *et al*, 1981) low levels of which are most closely related to the risk of atherosclerosis. So HDL levels certainly do not explain the predisposition of insulin dependent diabetics to large vessel disease.

The pathophysiology of the altered lipid and lipoprotein levels in diabetes mellitus has received considerable attention. LDL binding to its cell surface receptor and its subsequent internalisation and degradation is stimulated by physiological concentrations of insulin in tissue culture cells (Chait *et al*, 1978) probably by increasing receptor numbers rather than receptor affinity (Chait *et al*, 1979). Recent studies using the euglycaemic clamp technique support these findings in that LDL degradation by peripheral blood mononuclear cells was stimulated following a 4 h insulin infusion of 1 mU/kg/min in healthy young men (Mazzone *et al*, 1984). In this study there was also accelerated clearance of [^{125}I] LDL from plasma. In addition to possible effects of the diabetic state on LDL receptor activity it appears that LDL apoprotein B is susceptible to glycosylation and glycosylated LDL shows reduced binding to the LDL receptor (Gonen *et al*, 1981; Witztum *et al*, 1982). Modification of 5% or less of the lysine residues of LDL apoprotein B led to decreased LDL catabolism (Steinbrecher *et al*, 1984).

Different pathophysiological mechanisms may be responsible for raised VLDL-triglyceride concentrations depending on the type of diabetes. Insulin

dependent diabetics may have defective clearance of VLDL from the circulation (Nikkila and Kekki, 1973; Bagdade *et al*, 1967). Lipoprotein lipase, the enzyme responsible for the hydrolysis of VLDL triglyceride, depends for its full activity on adequate insulin concentrations and in insulin deficiency lipase levels are reduced (Nikkila *et al*, 1977; Jones *et al*, 1966). In addition increased production of VLDL has been reported (Nikkila and Kekki, 1973) and in some instances especially with severe hypertriglyceridaemia an underlying primary hypertriglyceridaemia may be present (Brunzell *et al*, 1975; Chait *et al*, 1981). In non-insulin dependent diabetic subjects the pathophysiology of hypertriglyceridaemia is more complex and both decreased catabolism of VLDL (Lewis *et al*, 1972) and over production (Kissebah *et al*, 1982; Abrams *et al*, 1982) have been described. It is probable that VLDL production is increased when free fatty acid levels are increased (Nikkila and Kekki, 1973; Abrams *et al*, 1982) and normal when free fatty acids are not increased (Howard *et al*, 1983).

Differences in HDL concentrations among different types of diabetic patients may be in part a reflection of the activity of the enzyme lipoprotein lipase (Nikkila, 1978; Nikkila *et al*, 1978) as many of the components of circulating HDL are not secretory products but are derived from transfer of apoproteins, phospholipids and triglycerides from triglyceride-rich lipoproteins and the esterification of free cholesterol by the LCAT enzyme (Nichols and Smith, 1965; Chajek and Eisenberg, 1978). Therefore one possible determinant of plasma HDL levels is the rate of catabolism of triglyceride-rich lipoproteins and indeed correlations have been described between HDL and lipoprotein lipase activities in both normal (Nikkila *et al*, 1978) and diabetic subjects (Nikkila and Hormila, 1978; Nikkila, 1978). Recently it has been demonstrated that HDL can be glycosylated (Schleicher *et al*, 1981; Witztum *et al*, 1982). However the significance of this finding remains to be determined.

Lipid and lipoprotein abnormalities do not explain the increased prevalence of vascular complications in the diabetic population. In fact all known risk factors taken together do not fully explain this increased risk. However lipid and lipoprotein abnormalities are certainly closely associated with vascular disease in diabetics as they are in non-diabetics. Mean fasting triglyceride concentrations were significantly higher in diabetics with complications than in those without (New *et al*, 1962). No difference was seen in mean cholesterol concentration. However in this study complications included nephropathy, retinopathy and neuropathy as well as large vessel disease. Fasting triglyceride and cholesterol concentrations have been analysed in relation to diabetics with and without large vessel disease (Santen *et al*, 1972). The groups were reasonably well matched for age, type of treatment and duration of diabetes but smoking habits were not assessed. Diabetics with large vessel disease had significantly higher plasma triglycerides than controls in the age range 30-59 years and a similar trend was seen for the age group 60-69 years. Cholesterol levels were higher in the younger age group with complications but the relationship was not as strong as that for triglyceride (Santen *et al*, 1972). However other studies have emphasised the relationship between cholesterol levels and vascular disease in diabetics (Lamba *et al*, 1974) but some studies have found no association with either cholesterol or triglyceride levels and vascular complications in diabetics (Elkeles *et al*, 1971). More recently relationships have been sought between individual lipoprotein concentrations and vascular disease in diabetics because in the general population lipoprotein levels are better predictors of vascular risk than total lipid levels. In a cross-sectional study of 154 diabetics (48 non-insulin dependent and 106 insulin dependent subjects) clinical evidence of large vessel disease was more likely to be present when lipid and lipoprotein concentrations were

high (Reckless *et al*, 1978) and the previously reported strong associations between triglyceride concentrations and the presence of vascular disease were supported the same relationships being found for VLDL. However these associations disappeared after an analysis of variance. This has also been emphasised in not-diabetic populations and may be explained by the inverse association between VLDL and HDL (Hulley *et al*, 1980). However significant associations with vascular disease remained after the analysis of variance for LDL-cholesterol and HDL-cholesterol concentrations but these associations were not present in all diabetic sub-groups. LDL-cholesterol concentrations remained positively associated with vascular disease in male diabetic patients on insulin whereas HDL concentrations remained negatively associated with vascular disease in all female diabetics (Reckless *et al*, 1978). The relationship of HDL to vascular disease in female diabetics has also been demonstrated in a prospective study where the especially high incidence of coronary artery disease in diabetic women with HDL-cholesterol levels below 0.88 mmol/l was emphasised. In these circumstances the apparent protection of women from the development of coronary artery disease compared to men is lost (Gordon *et al*, 1977).

Platelets

Introduction

Platelets which, like red blood cells, do not contain a nucleus, are the smallest of the formed elements of blood circulating as biconcave discs approximately 2-3 μm in diameter. They are formed from megakaryocytes which in the final process of maturation become amoeboid in shape and pseudopods penetrate the marrow sinusoids. These pseudopods fragment in the blood flow with the nucleus remaining in the bone marrow (Penington, 1981). The normal circulating platelet count varies in man between 150 and 400 x 10^9/l (Dacie and Lewis, 1975) and mean platelet survival is about 9.9 days (Harker and Finch, 1969). Platelets are removed from the circulation in the spleen and liver (Aster, 1969).

Platelets have a central role in normal haemostasis. Through their properties of adherence to vascular or foreign surfaces and their aggregation with other platelets they can initiate haemostasis. Platelets also participate in the fluid phase of coagulation through their active biochemical contents and by providing surfaces for reactions to take place. Platelets may also contribute to the development of atherosclerosis.

Historical Aspects

Robb-Smith (1967) has provided a fascinating account of the early descriptions of blood platelets which depended on the development of microscopic techniques of sufficient resolution. From Robb-Smith's researches it is probable that it was George Gulliver in 1841 who made the earliest representations of blood platelets which were included in an appendix and notes to an English translation of Gerber's "General and Minute Anatomy". However Gulliver made it clear that he did not think

that platelets were associated with fibrin formation. On the other hand William Addison an almost exact contemporary of Gulliver described the 'minute molecules' or 'granules' in relation to the formation of 'delicate and perfectly cylindrical filaments or fibres ....'. Addison's article 'On the Colourless Corpuscles and on the Molecules and Cytoblasts in the Blood' was accompanied by illustrations which leave no doubt that he was observing a platelet-fibrin clot. Franz Simon, a Berlin biochemist performed microscopic studies in blood treated with ferrocyanide to prevent clotting and saw numerous 'small bodies' (almost certainly platelets). This observation was repeated by Gustav Zimmermann, a German military physician who observed 'billions' of small colourless bodies which he called elementary bodies. Robb-Smith (1967) points out the scathing attack made on these findings by Rudolf Virchow in 'Die Cellularpathologie' which appeared in 1858, Virchow himself suggesting that the elementary bodies were artifacts. Virchow of course made a major contribution to pathology with the concept of thrombosis and embolism but an English physician, William Kirkes first recognized the nature of arterial embolism and infarction in 1852 and an English opthalmic surgeon Thomas Wharton Jones described the stages of thrombosis. Jones in an essay 'On the State of the Blood and Blood Vessels in Inflammation' observed 'arteries becoming blocked up by a mass composed apparently of colourless corpuscles and fibrin .... By and by the plug was pushed along in the artery by the force from behind and the flow of blood was re-established down to the first considerable branch above this place where the plug again stopped. A portion of the plug of granulous substance becoming detached and carried away, the mass was reduced in size so that it admitted of being again forced on .... I have found that on pressing the web over an artery or vein - a large vein especially - pretty firmly with a blunt point, an agglomeration of colourless corpuscles with a few

red ones, held together apparently by coagulated fibrin, occurs, adheres to the wall of the vessel and more or less completely obstructs it at the place .... In some cases I have been able to satisfy myself that the plugs of grey granulous substance consisted of colourless corpuscles, agglomerated and held together by a tenacious looking matter, probably coagulated fibrin. In other cases it appeared to me that the grey substance consisted of minute granules, held together by the tenacious matter'. This account of observations in a traumatised vessel in the web of a frog's foot is clearly the formation of fibrin platelet thrombi. Other important contributions in the development of the understanding of the nature of platelets and their relationship to thrombosis followed. Professor Max Schultze of Bonn in 1865 developed a method for keeping blood warm during microscopic analysis and observed the coalescence of platelets into granular masses (Koernchenhaufen), and in addition he appreciated the relationship of platelets to fibrin formation. William Osler working at University College also used this technique and in 1874 submitted a paper entitled 'An account of Certain Organisms Occurring in the Liquor Sanguinus' to the Royal Society. In this paper he reviewed previous work and gave a detailed account of individual platelets. In studies of blood vessels in loose connective tissue of the rat he described individual platelets with no tendency to stick together, but never granular masses. However if blood was taken from the animal granular masses formed. He also studied the formation of granular masses and observed the formation of pseudopods at the periphery of the mass associated with fibrin production. Giulio Bizzozero who was Professor of General Pathology in Turin made important contributions to the study of thrombosis and published in 1882 'On a New Element of Mammalian Blood and Part it Plays in the Production of Thrombi and Coagulation Generally'. In addition to repeating earlier work he showed that it was platelets that formed the

first stage of thrombosis and demonstrated their relationship to coagulation. In 1881 Osler observed that the white thrombus on an atheromatous plaque consisted entirely of platelets and in 1885 Kurt Schimmelbusch described the morphological changes occurring on contact of platelets with foreign surfaces or a damaged vessel and the increased stickiness of individual platelets and he believed that these events preceded fibrin formation. In 1906 Wright determined the relationship of platelets to the mekakaryocyte (reviewed by Robb-Smith, 1967).

These findings in the latter half of the nineteenth century reflected the increasing power of the optical microscope and subsequent progress in this century has depended on the application of the electron microscope to the analysis of platelet ultrastructure (reviewed by White *et al*, 1981); increased understanding of platelet biochemistry (reviewed by Gordon, 1981); the ability to measure platelet function (Born, 1962); appreciation of the importance of arachidonic acid metabolites in platelets and platelet vessel wall interactions (reviewed by Moncada, 1983) and the relationship of platelets to atherogenesis (Ross and Glomset, 1976).

Platelet Structure

Platelet structure and its relationship to platelet function has been given tremendous impetus through the application of electronmicroscopy to the study of platelet anatomy and the development of techniques for isolation of physiologically active platelets and their preservation for ultrastructural study (David-Ferreira, 1964; White, 1979). The subject has been reviewed (White *et al*, 1981) and will be briefly described here (see Figure 1). Platelet anatomy is generally considered in four zones for simplicity and also to relate structural to physiological activities.

The peripheral zone comprises the platelet cell surface, the surface connected open canilicular system, the exterior coat or glycocalyx, the

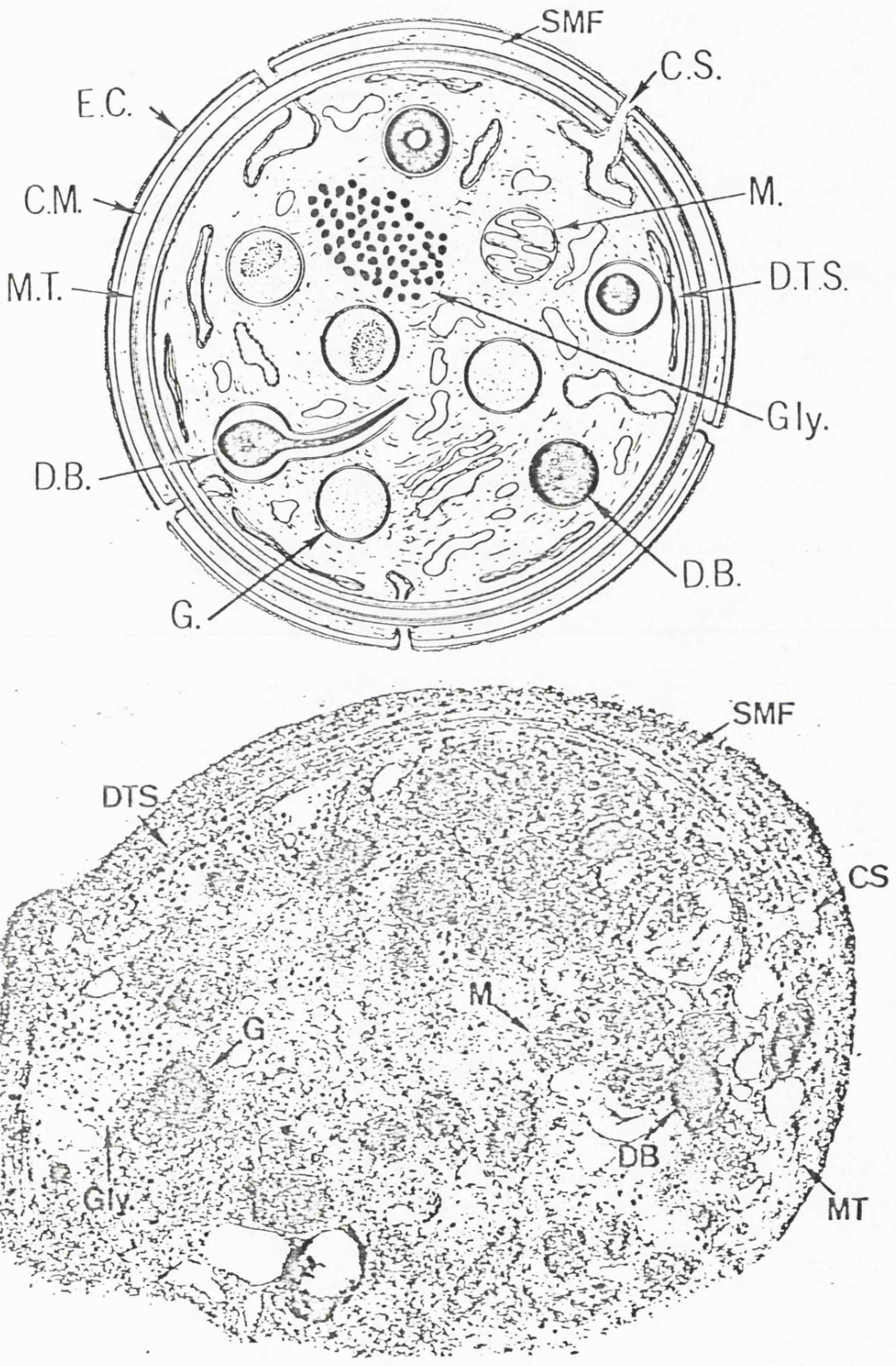

Figure 1. Platelet Ultrastructure (White *et al*, 1981).

EC, exterior coat; CM, trilaminar unit membrane; SMF, sub-membrane filaments; CS, surface connected cannilicular system; MT, microtubules; Gly, glycogen; M, mitochondria; G, granules; DB, dense bodies; DTS, dense tubular system.

unit membrane and the sub-membrane region. The glycocalyx of platelets is thicker and more dense than surface coats of other blood cells and is rich in glycoproteins (Berndt and Phillips, 1981) which is not surprising bearing in mind the number of receptor functions and transport mechanisms active at the platelet surface. The identification and analysis of this large number of glycoproteins has depended on the use of a large number of surface labeling probes such as lactoperoxidase catalysed iodination (Phillips, 1972) and transglutaminase (Okumura and Jamieson, 1976) which label amino acids and galactose oxidase which labels carbohydrate (Phillips and Agin, 1977). In addition to these enzyme catalyzed labelling techniques membrane impermeable reagents have been used including [^{203}Hg]p-chloro-mercuribenzene sulphonate (Nachman and Ferris, 1972), diazotized [^{125}I] diiodosulphanilic acid (George *et al*, 1977), pyridoxal phosphate with subsequent reduction of the Schiff's base with [^{3}H] sodium borohydride (McGregor *et al*, 1979) and the oxidation of sialic acid residues with sodium metaperiodate and reduction with [^{3}H] sodium borohydride (Gahmberg and Andersson, 1977). Following the use of these labelling techniques various electophoretic analyses has enabled the separation and identification of the labeled proteins including one dimensional SDS-polyacrylamide gel electrophoresis (Jenkins *et al*, 1980), two dimensional gel analysis (O'Farrell, 1975), two dimensional non-reduced - reduced SDS -polyacrylamide gel electrophoresis (Phillips and Agin, 1977b) and crossed immunoelectro-phoresis (Hagen *et al*, 1980). Although these techniques have identified many glycoproteins on the platelet surface (> 30) there are some notably Ib IIb III and IV which are present in high concentration and their functional roles are emerging as will be described later.

The trilaminar unit membrane of the peripheral zone which provides the physiochemical separation between extracellular and intracellular constituents has important components including the sodium/potassium ATPase.

There do not appear to be major differences between the platelet membrane and that of other blood cells. However intercalated particles (probably trans-membrane proteins) appear to be fewer in number (White, 1979). The membrane lipids, important as sources of arachidonic acid for the cyclo-oxygenase reaction are distributed assymetrically with sphingomyelin and phosphatidyl choline on the outside and phosphatidyl inositol, phosphotidyl ethanolamine and phosphatidylserine on the inner surface (Schick, 1979). The sub-membrane region contains a regular system of filamentous elements (White, 1969) which can be seen lateral to the circumferential band of microtubules in discoid platelets (Zucker-Franklin, 1970). These filaments which are in close association with the cell membrane may be concerned with the maintenance of platelet shape and in pseudopod formation in conjunction with actin binding protein and α-actinin (Lucas *et al*, 1976; Schollmeyer *et al*, 1978).

The so-called sol-gel zone of platelets contains the important microtubules and microfilaments. Microtubules of which tubulin is the major protein constituent are located in a circumferential band in the equatorial plane just under the cell wall in discoid platelets, which suggests that they may be important in maintenance of the platelet cytoskeleton. In addition, factors (such as chilling or exposure to agents like colchicine and vinca alkaloids which disrupt or prevent formation of microtubules) which produce loss of the discoid shape of platelets are associated with loss of the band of microtubules (White and Krivit, 1967; White, 1968). In addition microtubules have a role in platelet contraction when platelets are activated, but depolymerization of the tubules does not affect the ability of platelets to contract and cause clot retraction (White, 1969b). However there was a transient decrease in polymerized tubulin a few seconds after platelet activation induced by a variety of aggregatory agonists which recovered after 1-4 min (Steiner and Ikeda, 1979) and following platelet

activation microtubules were observed in a tight band around centrally grouped organelles (White, 1968b). Therefore microtubules may be involved in internal alignment of platelet organelles prior to the release reaction and furthermore may help govern the degree of response to circulating agonists (White *et al*, 1981).

The second system of fibres in the sol-gel zone of platelets comprises the microfilaments (White, 1971). They resemble actin filaments found in other contractile cells such as muscle and platelet actin has a hundred-fold excess over platelet myosin (Pollard, 1975). The microfilament system provides the contractile capacity for the release reaction seen in activated platelets (Adelstein and Pollard, 1978). Cohen *et al* (1980) have postulated that the actin filaments are anchored to the platelet membrane by α-actinin (glycoprotein III) a trans-membrane protein (Gerrard *et al*, 1979). The actin filaments extend into the cytoplasm from this point and form long filaments orientated concentrically around the platelet granules. The myosin filaments would interdigitate between the actin filaments in opposite directions and help to push the platelet granules into the centre of the platelet for release into the surface connected open canalicular system.

The platelet organelle zone is rich in several types of organelle. In addition to mitochondria platelet cytoplasm contains numerous granules and dense bodies which have been distinguished by density, ultrastructural studies and biochemical content. The dense bodies (≃ 250-300 nm) so-called because of the electron dense opaque internal content when visualized with the electronmicroscope are the storage sites for 5-hydroxytryptamine, adenine nucleotides, calcium and pyrophosphate (Holmsen and Weiss, 1979). Alpha-granules are the most numerous of platelet organelles and they have a variety of densities and staining characteristics. These granules (300-500 nm) store many proteins some of which are specific to platelets

such as platelet factor 4, β-thromboglobulin, low affinity platelet factor 4 and platelet derived growth factor. In addition alpha-granules contain proteins which are found in other cells and plasma such as fibrinogen, fibronectin, albumin and factor VIII-related antigen. It is probable that there are different types of alpha-granule with different granular contents which may be released in different situations (Kaplan, 1981). Platelets also possess a variety of lysosomal enzyme storage organelles containing acid hydrolases including β-glucuronidase, β-N-acetylglucosaminidase and β-galactosidase (Holmsen, 1975).

Platelets contain two important membrane systems the surface-connected open canalicular system and the dense tubular system. The canalicular system consists of tortuous invaginations of the platelet wall running through the platelet cytoplasm and therefore greatly increasing the total surface area of platelet/plasma contact (Behnke, 1970). This system remains patent despite platelet contraction, adhesion and aggregation and probably serves for the release of the contents of the platelet storage granules to plasma (White, 1973). The dense tubular system which probably represents residual smooth endoplasmic reticulum is discrete from the open canalicular system (Behnke, 1970) but forms very close relationships with it in certain areas of the cytoplasm (White, 1972). An important physiologic role for the dense tubular system is calcium sequestration through the activity of the calcium/magnesium ATPase (Cutler *et al*, 1978) as will be discussed later.

Platelet Physiology

Haemostasis

A series of complex reactions involving the vessel wall, plasma proteins and blood platelets occur to reduce the loss of blood following injury to the vasculature. Platelets contribute to these complex reactions

in three main ways. They adhere to exposed collagen and form a physical plug to occlude the wound; they release active substances from their secretory granules which help to consolidate the initial platelet plug; they play an important role in interacting with the coagulation system and certain intrinsic coagulation reactions occur preferentially on the platelet surface. The process of platelet plug formation has been reviewed by Sixma and Wester (1977). Macroscopically observations of haemostatic plug formation have been made in animal models particularly the hamster cheek pouch and the rabbit mesentery. Whereas transection of a capillary causes no bleeding, arterial transection results in contraction, retraction and brisk bleeding. Contraction is probably only important in the larger arteries and no contraction or retraction is seen when a vein is transected. Immediately following transection of the blood vessel there is accumulation of platelets on the exposed connective tissues at the edge of the wound. This is seen as a greyish-white accumulation of material which grows and obstructs blood flow. Eventually bleeding stops but may restart through channels in the platelet plug which are then occluded again. The haemostatic plug protrudes from the vessel and the blood behind the plug becomes stagnant.

Histological studies of platelet plug formation have been performed in animals and in biopsies of human skin following bleeding time studies (Sixma and Wester, 1977; Wester *et al*, 1978; Wester *et al*, 1979). The earliest finding is the platelet adhesion to collagen fibres followed by aggregation and formation of a platelet plug. There is some fibrin formed at the edges of the wound and at the plug periphery but no fibrin is seen within the plug for about 15-30 min. In the first minute of the plug formation the platelets retain their granules and remain loosely packed. However subsequently they become more closely packed and release their granules and the plug becomes a dense mass of inter-

digitated platelets. The major changes to the plugs in the hours following transection of the vessel are that lytic areas initially seen at the edges of the plug also develop in its centre and the degranulated platelets resemble empty vesicles. The pseudopodia disappear and the platelets appear round once more (Wester *et al*, 1979). In addition there is increased fibrin formation with masses of fibrin between the platelets eventually forming a network of thick strands after 24 h (Hovig *et al*, 1968). The platelet plays an important role in interacting with the coagulation system and certain intrinsic coagulation reactions occur preferentially on the platelet surface which appears to protect coagulation enzymes from inactivation by plasma proteinases and localises fibrin formation within and around the platelet plug (Walsh, 1981). Fibrin deposition strengthens the platelet plug and the haemostatic barrier is complete. There is also infiltration of polymorphonuclear and mononuclear cells in the wound and sometimes in the plug itself and interestingly along the blood vessel near the wound (Wester *et al*, 1979).

As can be seen from the above description the formation of the haemostatic plug or "primary haemostasis" involves many properties of platelets including adhesion, aggregation, degranulation and release reaction and these platelet properties will be briefly described.

Platelet Adhesion

It is thought that the platelet is necessary for the maintenance of normal vascular integrity in that patients with thrombocytopaenia show a generalised haemorrhagic tendency and bruising when the platelet count falls below about 50 x 10^9/l, spontaneous bruising and more severe bleeding being possible as the platelet count falls below 20 x 10^9/l. In addition haemorrhage can also occur due to qualitative platelet defects such as essential thrombocythaemia (McClure *et al*, 1966). The mechanism for the

maintenance of this functional integrity is yet to be established but presumably would involve platelet adhesion to the endothelium.

Platelets do not usually adhere to normal endothelium but the reasons why normal endothelium is inert with respect to stimulating platelet adhesion and aggregation is not fully understood (Shattil and Bennett, 1981). However Moncada *et al* (1976) demonstrated that endothelial cells were capable of producing a potent anti-platelet aggregatory agent and it was subsequently suggested that continuous production of this substance now called prostacyclin explained the apparent protection against adhesion and aggregation conferred by the normal endothelium (Moncada *et al*, 1976; Moncada and Vane, 1979). The ability of vascular endothelium to inhibit platelet aggregation had been previously noted (Saba and Mason, 1974) but the presence of an ADP-ase enzyme had been suggested to explain this observation (Heyns *et al*, 1974). Subsequent experimental work has cast doubt on this suggestion. For instance in the rabbit, doses of aspirin sufficient to inhibit prostacyclin production does not lead to platelet adhesion to intact endothelium (Dejana *et al*, 1980). In addition several studies have demonstrated that the amounts of prostacyclin normally found in the circulation are probably too low to have an appreciable effect on platelets (Steer *et al*, 1980; Haslam and McClenaghan, 1981; Greaves and Preston, 1982) and endothelial cells in culture produce little prostacyclin unless stimulated by thrombin for instance (Weksler *et al*, 1977).

Platelets will adhere to a variety of surfaces, both artificial and naturally occurring, the most important *in vivo* is the adhesion to collagen which occurs when the blood vessel endothelium is damaged. This will be discussed in a later section of this thesis.

Platelet Aggregation

Platelet aggregation or platelet-platelet stickiness can be regarded as a special case of platelet adhesion and follows adhesion of platelets

to injured surfaces. Normally, as has been described, aggregation is apparent in experimental wounds within seconds. Aggregation has been studied *in vitro* more than any other platelet activity because of the development of a simple photometric method for its measurement (Born, 1962). This is based on the observation that the optical density of stirred platelet-rich plasma (PRP) falls as aggregates develop and returns to normal as they disperse. Aggregation *in vitro* which is an active process (Vargaftig *et al*, 1981) is dependent on pH, temperature, mechanical stirring, calcium ion concentration, the concentration of platelets in the PRP and the time elapsed between the performance of the aggregation tests and the preparation of the PRP (Mills, 1981). Numerous natural and artificial substances are capable of causing platelet aggregation Table 3) and depending on the nature of the aggregating agent and its concentration, aggregation may be reversible or irreversible and have one or two phases.

Adenosine diphosphate (ADP) was the first agent discovered that caused aggregation (Gaarder *et al*, 1961). ADP induces aggregation directly and leads to the platelet release reaction (Macmillan, 1966). A single phase of aggregation followed by rapid disaggregation is seen with low concentrations of ADP. At threshold concentrations a second phase caused by ADP release from the platelets is seen and at high concentrations a single phase with irreversible aggregation is observed (Hardisty *et al*, 1970). Primary ADP-induced aggregation requires fibrinogen (Niewiarowski *et al*, 1977) which appears to be involved in early transitory interactions between platelets (Mustard *et al*, 1978). Other plasma protein co-factors, possibly adsorbed coagulation factors of the vitamin K dependent group may also be required (Miale and Kent, 1975). ADP may play a central role in the initiation of physiological platelet aggregation. This nucleotide is extruded from activated platelets and also may be derived from injured

Table 3

Platelet Aggregating Agents

Adenosine 5'diphosphate	Gaarder *et al*, 1961
Collagen	Zucker and Borelli, 1962
Thrombin	Grette, 1962
Adrenaline	Mitchell and Sharpe, 1964
5-Hydroxytryptamine	Mitchell and Sharpe, 1964
Immune complexes	Mueller-Eckhardt and Luscher, 1968
Platelet activating factor	Beneviste *et al*, 1972
Thromboxane A_2	Hamberg *et al*, 1975
Sodium Arachidonate	Kinlough-Rathbone *et al*, 1976
Calcium ionophores	Massini and Luscher, 1974

tissue and erythrocytes (Bergvist and Arfos, 1976).

At low concentrations of collagen platelet aggregation is dependent on the release of arachidonic acid from platelet membrane phospholipid and the subsequent metabolism of arachidonic acid via the prostaglandin pathway (Packham, 1976). However at higher concentrations collagen also causes release of ADP from platelet granules and this is independent of the prostaglandin pathway. For collagen to promote platelet aggregation it must possess its triple helical structure and must be present as fibrillar collagen (Zucker and Borelli, 1962).

Adrenaline induces a biphasic response when added to PRP (Macmillan, 1966). The first wave of aggregation induced by adrenaline is associated with uptake of calcium by platelets and it has been suggested that a localized flux of calcium into the platelet plasma membrane enables the platelets to stick together (Gerrard *et al*, 1981). Unlike other aggregating agents (ADP, collagen and thrombin) adrenaline does not cause initial platelet shape change (seen as a transient increase in optical density of PRP). These other aggregating agents which cause shape change before platelet-platelet aggregation release calcium initially from an intracellular structure probably the dense tubular system so that there is an elevation in cytoplasmic calcium before plasma membrane calcium is altered (Gerrard *et al*, 1981).

The mechanisms by which platelet sticks to platelet during aggregation are not fully understood. However, extracellular calcium is necessary and there is considerable evidence particularly from studies of thrombasthenic platelets that the platelet surface glycoproteins IIb and III are involved (Nurden and Caen, 1974; Nurden and Caen, 1975; Phillips and Agin, 1977). More recently, fibrinogen has been strongly implicated in platelet/platelet interactions. Platelets activated by thrombin were found to produce haemagglutinin activity with fixed bovine erythrocytes

(Gartner *et al*, 1978) and subsequently the same group have shown that this agglutinin is bound to platelet membranes and appears in the incubation fluid following platelet activation by thrombin (Gartner *et al*, 1980). It is highly likely that this agglutinin is fibrinogen (Gartner *et al*, 1980; Berndt and Phillips, 1981). Indeed the role of fibrinogen in platelet aggregation has been recognised for many years (Born and Cross, 1964; Brinkhous *et al*, 1965). ADP stimulated platelets exhibit increased fibrinogen binding (Mustard *et al*, 1978) and the features of the binding suggested a fibrinogen receptor on the platelet membrane which required extracellular calcium (Bennett and Vilaire, 1979; Peerschke *et al*, 1980).

Platelet Release Reaction

The release of platelet-derived material and the profound biochemical and morphological changes that occur after platelet stimulation have been termed the platelet release reaction (Grette, 1962). On stimulation platelets release their dense bodies, alpha granules and lysosome-like granules (see Table 4) but the integrity of the membrane structures, cytoplasm and mitochondria is preserved. Grette performed his experiments in porcine platelets stimulated by thrombin but subsequently many substances (secretagogues) have been found to provoke platelet aggregation. ADP, adrenaline and collagen as well as thrombin provoked release of 5-hydroxytryptamine and nucleotides from platelets (Mills *et al*, 1968). In addition immune complexes (Humphrey and Jaques, 1955) arachidonic acid (Kaplan *et al*, 1979) and its metabolites prostaglandin endoperoxides and thromboxane A_2 (Hamberg *et al*, 1975) cause platelet release. More recently the degranulating action of platelet-activating factor has been demonstrated (Chignard *et al*, 1979; MaManus *et al*, 1979). Some secretagogues (e.g. ADP) stimulate the release of certain granules (dense bodies, some alpha granules but not the lysosomal granules) whereas thrombin

Table 4. Subcellular localization of secreted platelet constituents

Alpha-granules	Dense granules	Lysosomes
Factor V Fibrinogen Albumin Fibronectin Platelet factor 4 Low affinity platelet Factor 4/beta thromboglobulin Platelet derived growth factors	ATP ADP Pyrophosphate 5-hydroxytryptamine Calcium	Acid hydrolases

stimulates secretion of all types of granule including lysosomes (Holmsen, 1977). In addition to these 'physiological stimuli' many 'non-physiological' stimuli stimulate platelet degranulation including calcium ionophores (Gerrard *et al*, 1974), lectins (Greenberg and Jamieson, 1974) and latex and viral particles (Holmsen, 1977). As reviewed by Skaer (1981) it is probable that there are many different types of granule. In addition the response to various secretagogues in terms of which granules are released and to what extent varies which suggests that platelets must possess very sophisticated intracellular control mechanisms.

Dense body contents are discharged through the platelet surface membrane (Skaer, 1981) and this occurs from between 4 sec and 3 min after addition of the secretagogue. Mobilisation of the internal calcium occurs after 0.75 - 1.8 sec following the addition of thrombin (Feinstein, 1980). Therefore the release of intracellular membrane bound calcium precedes the onset of the stimulus induced degranulation. A further factor involved in the stimulus to degranulation may be the breakdown of phosphatidylinositol with the accumulation of diglyceride (1-stearoyl,2-arachidonyl diglyceride). Diglyceride levels after thrombin stimulation increase 30-fold after 15 sec (Rittenhouse-Simmons, 1979). The diglyceride may be important in promoting membrane fusion of the dense body granule with the platelet surface membrane and also as a substrate for the formation of prostaglandin endoperoxides and thromboxane A_2 which are powerful degranulation stimuli (Bell *et al*, 1979). In addition the actual breakdown of the membrane polyphosphoinosides by calcium stimulated enzymes may promote degranulation as they have highly charged polar head groups which would tend to impede membrane fusion (Allan and Michell, 1979).

As has been seen the platelet alpha-granules contain a large number of proteins and are very numerous in the platelet cytoplasm. For instance

alpha-granule fibrinogen forms approximately 10% of total platelet protein (Holmsen and Wens, 1979). Not all alpha-granules may respond in the same way to secretory stimuli in that Zucker *et al* (1979) who demonstrated that over 80% of platelet factor VIII related antigen was present in the alpha-granules could only demonstrate the release of 30% of this in response to stimulation with collagen. Similar findings have also been described for fibronectin (Ginsberg *et al*, 1979). Although there are several possible explanations for these findings as reviewed by Skaer (1981) it is possible that some alpha-granules may remain refractory to secretory stimuli.

The contents of the alpha-granules unlike those of the dense bodies are discharged into the open canalicular system. Alpha-granule nucleoids have been demonstrated by electronmicroscopic studies in the canalicular system (White, 1974) and it is likely that normal secretion involves not only fusion of these granules with the canalicular system but in addition platelet contraction, the evidence for this coming from the use of differing secretagogues (Gerrard *et al*, 1974).

Platelet Prostaglandin Metabolism

Over recent years there has been increasing interest in these widely distributed and biologically important compounds and their importance in platelet biochemistry and platelet/vessel wall interactions has been emphasized.

The prostaglandins and thromboxanes are produced from certain 20-carbon atom polyunsaturated fatty acids with varying degrees of unsaturation. The most abundant of these acids in human tissue is arachidonic acid (Crawford, 1983). Most arachidonic acid is esterified in membrane phospholipid complexes and in order to act as a substrate for the enzyme complex, prostaglandin synthetase, the free

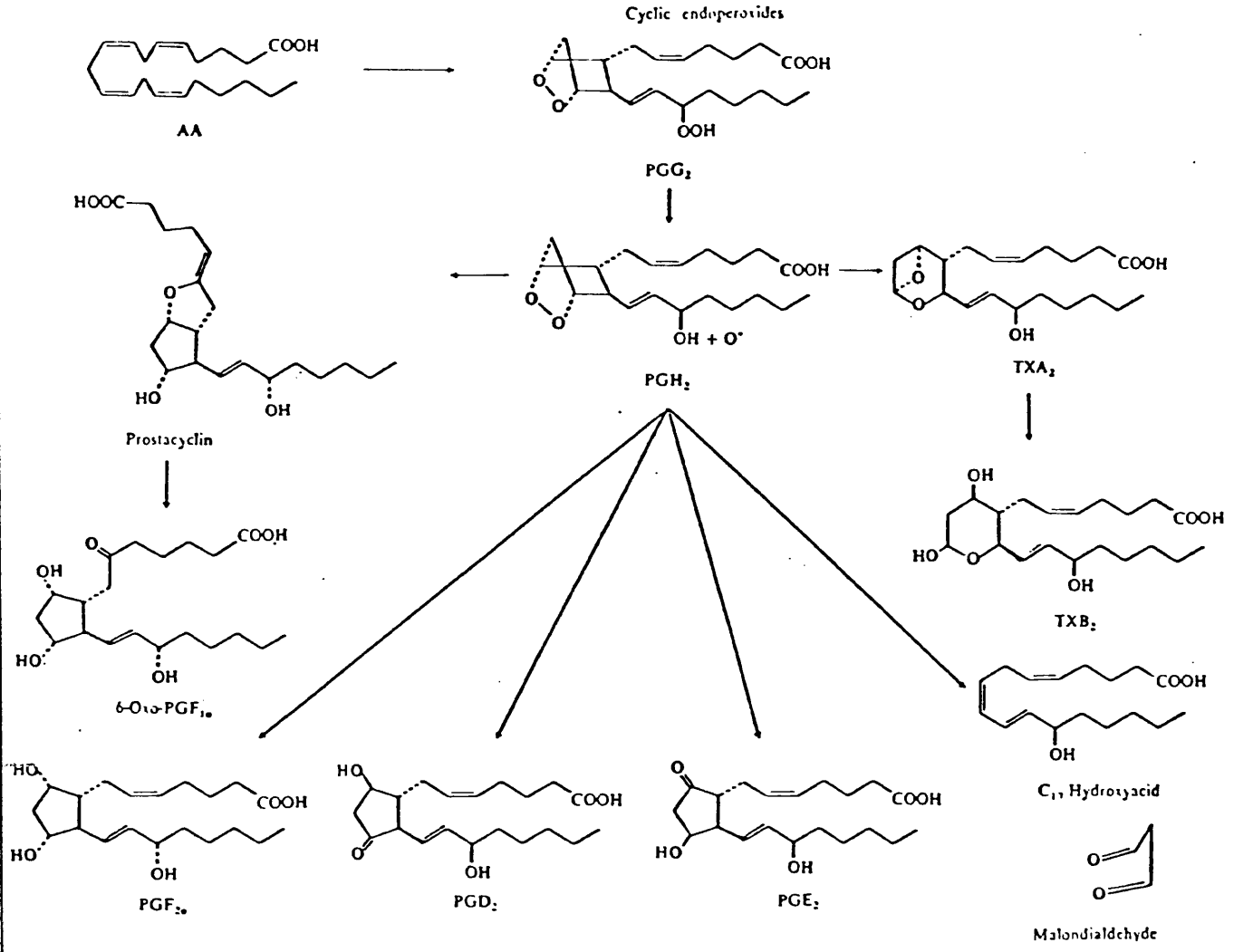

Figure 2. The prostaglandin pathway (from Moncada, 1983). Arachidonic acid, AA; prostglandin, PG; thromboxane, TX.

acid must be liberated by the action of phospholipase A_2. The activity of this membrane enzyme thus provides the first control step in the prostaglandin pathway by limiting the availability of arachidonic acid substrate.

Free arachidonic acid is then acted upon by the system of microsomal enzymes known as prostaglandin synthetase which is composed of cyclo-oxygenase and peroxidase activities. In the presence of molecular oxygen, the cyclo-oxygenase activity converts arachidonic acid to a 15-hydroperoxide endoperoxide known as prostaglandin G_2 which is then converted by the peroxidase activity to a 15-hydroxy endoperoxide known as prostaglandin H_2 (Lands, 1979)(see Figure 2). Prostaglandin H_2 is an unstable compound which has a half-life of approximately 5 min (van Dorp *et al*, 1978) and once formed is rapidly transformed either enzymatically or non-enzymatically to the classical prostaglandins, prostaglandin D_2, E_2 and $F_{2\alpha}$ (Nugteren and Hazelhof, 1973; Miyamoto *et al*, 1974; van Dorp *et al*, 1978). Most tissues are capable of synthesizing these unstable endoperoxides and prostaglandins and it is further products of the prostaglandin pathway which determine the biological effects observed in different tissues. The synthesis of the prostaglandins E_2 and $F_{2\alpha}$ was demonstrated in platelets stimulated with thrombin by Smith and Willis (1971). These authors found that oral administration of aspirin was associated with inhibition of platelet prostaglandin synthesis and suggested that this finding might explain the known effects of aspirin in inhibiting the platelet release reaction (O'Brien, 1968) and in prolonging the bleeding time (Quick, 1966). However it was difficult to accept this concept as the prostaglandins measured were not pro-aggregatory. This was resolved a short time later when an unstable product of a short term incubation of arachidonic acid with cyclo-oxygenase prepared from the vesicular gland of sheep was found to induce platelet aggregation (Willis and Kuhn, 1973). This product was

subsequently found to be prostaglandin H_2 and the unstable endoperoxides prostaglandin G_2 and prostaglandin H_2 were found to be released during platelet aggregation (Hamberg *et al*, 1974). In addition when these compcunds were added to platelets they provoked aggregation (Hamberg *et al*, 1974; Hamber and Samuelsson, 1974). Platelet aggregation induced by endoperoxides appeared to be greater than that which could be accounted for by the endoperoxides alone and Hamberg *et al* (1975) were able to demonstrate that in platelets, endoperoxides are further metabolised to a very unstable compound, thromboxane A_2. Thromboxane A_2 has a half-life of 30 sec at body temperature and degrades spontaneously to the stable metabolite thromboxane B_2 (Hamberg *et al*, 1975). Subsequently the microsomal fractions of human platelets have been shown to contain an enzyme which converts the endoperoxides, prostaglandin G_2 and H_2 to thromboxane A_2 and the enzyme has been designated thromboxane synthetase (Needleman *et al*, 1976). Thromboxane synthetase also catalyses the conversion of prostaglandin H_2 to a C-17 hydroxy acid known as 12-hydroxy-5.8.10-hepta-decatrienoic acid and malondialdehyde (Diczfalusy and Hammarström, 1977). However prostaglandin H_2 conversion to these compounds may also occur non-enzymatically (van Dorp *et al*, 1978). It appears from experiments in which platelet membrane fractions have been separated that thromboxane synthesis is associated with intracellular membranes known as dense tubular membranes (Carey *et al*, 1982).

Platelets can also metabolise archidonic acid by a lipoxygenase enzyme system to yield a variety of hydroxy acids via their respective hydroperoxide intermediates (Hamberg and Sammuelson, 1974; Nugteren, 1975). The biological activity of these compounds remains to be determined. However 12-L-hydroperoxy 5,8,10,14-eicosatetraenoic acid may inhibit prostacyclin synthetase and cyclco-oxygenase (Siegal *et al*, 1979).

In 1976, a new relatively inactive arachidonic acid metabolite characterised as 6-oxo-prostaglandin $F_{1\alpha}$ was isolated from several tissues including guinea-pig lung (Dawsen *et al*, 1976) and rat stomach (Pace-Asciak, 1976). In the same year Moncada *et al* (1976) reported the presence of an enzyme in aortic tissue which converted prostaglandin endoperoxides to a potent unstable inhibitor of platelet aggregation. This factor also relaxed arterial smooth muscle and was named prostaglandin X (Moncada *et al*, 1976). Prostaglandin X rapidly lost activity in neutral and acidic media with the formation of a relatively inert substance subsequently identified as 6-oxo-prostaglandin $F_{1\alpha}$ and prostaglandin X was identified as 9-deoxy-6, 9-epoxy-Δ^5-prostaglandin $F_{1\alpha}$ and renamed prostacyclin (Johnson *et al*, 1976). The microsomal enzyme converting prostaglandin H_2 to prostacyclin was named prostacyclin synthetase and prostacyclin was found to be the major product of arachidonic acid in the walls of arteries and veins in several animal species and man (Moncada and Vane, 1978). However prostacyclin synthetase although present in many tissues is not present in platelets (Moncada and Vane, 1979).

Thromboxane A_2 is a more powerful pro-aggregatory substance in platelets than the endoperoxides and it has been proposed that it is the arachidonic acid metabolite that mediates platelet aggregation and the release reaction (Hamberg *et al*, 1975). Whether platelet endoperoxides have a pro-aggregatory role in their own right or only when converted to thromboxane A_2 has been the subject of many studies (Bunting *et al*, 1983). However from experiments using thromboxane synthetase inhibitors it appears that when platelets are activated and the prostaglandin cascade is triggered by release of endogenous arachidonic acid then the endoperoxides generated exert their pro-aggregatory effects by their conversion to the more potent compound thromboxane A_2. However when thromboxane generation is suppressed it is probably that endoperoxides can be pro-aggregatory themselves (Bunting *et*

al, 1983).

It has been proposed that endoperoxides and thromboxane A_2 activate platelets by acting as calcium ionophores hence mobilising intracellular free calcium (Gerrard *et al*, 1978). However all prostaglandins that induce platelet aggregation cause a monophasic or reversible 'primary' response at low concentrations and a monophasic irreversible 'secondary' response at high concentrations. In this way the activation of platelets by prostaglandins resembles that produced by ADP and adrenaline. This is unlike the activation induced by the powerful stimulus of calcium ionophores in that prostaglandins induce platelet dense body secretion and alpha-granule secretion but not secretion of lysosomal hydrolases (MacIntyre, 1979). For this reason and others (reviewed by MacIntyre, 1981) including studies with antagonists, desensitisation experiments and measurement of calcium ionophore activity it is thought that platelet activation by prostaglandins and thromboxanes is receptor-mediated although a suitable radio-labelled ligand is not available for definitive demonstration (MacIntyre, 1981).

Recently Defreyn *et al* (1981) have demonstrated a deficiency of thromboxane formation and platelet aggregation in platelets from a patient with a familial bleeding tendency thus emphasizing the importance of thromboxane production in the physiology of platelet function.

Thromboxane A_2 is a powerful vasoactive compound and the activity of the rabbit aorta-contracting substance released from sensitized guinea-pig lungs described by Piper and Vane (1969) is accounted for by this substance (Hamberg *et al*, 1975). Because of its highly unstable nature various thromboxane A_2 generating systems have to be used in order to study its vasoactive properties. Using these techniques it has been shown that thromboxane A_2 is a potent contractor of isolated vessel segments from several species including coronary arteries from pig and cattle, human

umbilical artery and rabbit aorta (Moncada and Vane, 1979). In addition strips of bovine cerebral conductance arteries and human basilar arteries contract with thromboxane A_2 (Ellis *et al*, 1979; Boullin *et al*, 1979).

The effect of thromboxane A_2 on differing vascular preparations has also been studied *in vivo* using thromboxane synthesized *in vitro* by incubations of endoperoxide with platelet microsomes. Using this technique Dusting *et al* (1978) were able to show short term vasoconstriction in femoral and vascular beds of the dog following injection of the thromboxane close to the vessels.

Prostacyclin has powerful metabolic effects on blood platelets. It has the most potent effect of the naturally occurring prostaglandins in inhibiting platelet aggregation being 30-40 times more potent than prostaglandin E_1 and 10 times more potent than prostaglandin D_2 *in vitro* (Moncada *et al*, 1976; Gryglewski *et al*, 1976). Prostacyclin can also bring about disaggregation of platelet aggregates *in vitro* (Moncada *et al*, 1976; Ubatuba *et al*, 1979). In addition using animal models, prostacyclin applied locally has been shown to inhibit thrombus formation in response to ADP in the hamster cheek pouch microcirculation (Higgs *et al*, 1977) and given systemically, prostacyclin inhibited thrombus formation induced electrically in rabbit carotid artery (Ubatuba *et al*, 1979). Similar findings were observed with prostacyclin either applied locally or given systemically in the coronary artery of dog (Aiken *et al*, 1979).

When given systemically prostacyclin protected against sudden death (secondary to platelet aggregation) in the rabbit following intravenous injection of arachidonic acid (Bayer *et al*, 1979). Gryglewski *et al* (1978) incorporated collagen strips into extracorporeal circuits in animals and showed that prostacyclin brought about disaggregation of platelet aggregates formed on the collagen strips. Similarly prostacyclin inhibited platelet aggregation on rabbit sub-endothelium but only prevented platelet

adhesion at higher doses (Higgs *et al*, 1978). In man prostacyclin infusion inhibited platelet aggregation measured *ex vivo* at a threshold dosage of 2 ng/kg/min and with increasing doses there was increased inhibition of ADP-induced aggregation (Fitzgerald *et al*, 1979).

Although platelets cannot synthesize prostacyclin they do possess the enzyme 9-hydroxyprostaglandin dehydrogenase (Wong *et al*, 1980). This enzyme can transform 6-oxo-prostaglandin $F_{1\alpha}$, the stable hydrolysis product of prostacyclin into 6-oxo-prostaglandin E_1 which does possess anti-aggregating effects (Quilley *et al*, 1980). However the possible biological role of this metabolite in platelet function remains to be determined as plasma levels of 6-oxo-prostaglandin E_1 (measured by gas chromatography, mass spectrometry techniques) did not increase following intravenous infusion of prostacyclin (Jackson *et al*, 1982).

The potent inhibitory action of prostacyclin on platelet aggregation correlates with its ability to stimulate the platelet adenylate cyclase system thus increasing cyclic AMP levels within the cell (Tateson *et al*, 1977). Stimulation of adenylate cyclase follows binding of prostacyclin to a platelet membrane receptor which it shares with prostaglandin E_1 but is distinct from the prostaglandin D_2 receptor (Miller and Gorman, 1979). Shepherd *et al* (1983) have calculated that each human platelet has 4200 high affinity binding sites for prostacyclin with a dissociation constant of 16 nmol. In keeping with its stronger anti-platelet aggregatory effects compared to prostaglandin E_1, prostacyclin stimulation of adenylate cyclase activity is more potent and more sustained (Gorman *et al*, 1977). By enhancing platelet cyclic AMP levels through its stimulation of adenylate cyclase, prostacyclin can reduce cytoplasmic calcium levels which determine platelet shape change, aggregation and the release reaction (Käser-Glanzmann *et al*, 1977). In addition platelet phospholipase (Minkes *et al*, 1977) and cyclo-oxygenase (Malmsten *et al*, 1976) are inhibited by

the elevated cyclic AMP levels.

Prostacyclin is the major product of arachidonic acid in blood vessels (Moncada *et al*, 1977a) and the synthesis is highest in the intima (Moncada *et al*, 1977). Capillaries also generate prostacyclin (Goehlert *et al*, 1981) and studies with cultured cells have demonstrated that it is endothelial cells which are the main source (Weksler *et al*, 1977). Thus when endothelium was stripped from rabbit aorta *in vivo* prostacyclin production at the luminal surface of the vessel was virtually absent in response to arachidonic acid and recovery of prostacyclin production was slow over a period of 70 days coinciding with the appearance of neo-intimal cells (Eldor *et al*, 1981).

Prostacyclin in contrast to thromboxane A_2 produced relaxation in most arterial preparations tested *in vitro* and was a potent vasodilator when tested in isolated perfused heart preparations (reviewed by Moncada and Vane, 1979). In addition, Dusting *et al* (1979) demonstrated increased blood flow in the coronary arteries of the intact heart in open-chested dogs following application of prostacyclin. Prostacyclin is a potent vasodilator in the micro-circulation as demonstrated using the hamster cheek pouch preparation by Higgs *et al* (1982) and in the cerebral micro-circulation of the cat by Ellis *et al* (1979).

In intact experimental animals prostacyclin has a vasodepressor effect and this was observed to an equal degree when injected intravenously or intra-arterially (Armstrong *et al*, 1978). This work suggests that prostacyclin is not inactivated in the pulmonary circulation unlike other prostaglandins. However the vascular activity of prostacyclin *in vivo* is very short-lived and is less than its chemical half-life at physiological temperature and pH (i.e. < 3 min). This suggests that biological inactivation takes place very rapidly *in vivo* and most metabolites of prostacyclin have little vasodepressor activity apart from 6-oxo-prostaglandin E_1 (reviewed by Whittle and Moncada, 1983).

Platelets, Thrombosis and Atherogenesis

Thrombosis

Platelets are undoubtedly involved in the final occlusion of atheromatous arteries and form the major component (the "white head" of aggregated platelets) of the occlusive thrombus (Davies and Thomas, 1981). This sudden and often catastrophic event is most likely precipitated by fissuring of an underlying atheromatous plaque as detailed analysis of obstructed coronary arteries at post-mortem invariably show that the thrombus is found in association with haemorrhage into a plaque (Chapman, 1965; Constantinides, 1966 and Friedman, 1970). In addition to their apparent central role in the pathogenesis of the arterial thrombus increasing interest has focussed on platelets and the coronary microcirculation with the finding of platelet aggregates in the coronary microcirculation in some cases of sudden cardiac death (Haerem, 1972; El-Maraghi and Genton, 1980). These findings have stimulated an increasing amount of research into the mechanisms involved yet the pathogenesis of thrombus formation remains to be clarified. Certainly platelets do not normally adhere to arterial endothelium if it is undamaged but the explanation for this antithrombotic property of intact endothelium remains obscure. Following the discovery of prostacyclin it was suggested that endothelial cell production of this very potent anti-aggregatory substance might explain this important property (Moncada *et al*, 1977; Moncada and Vane, 1978). However this suggestion has not been confirmed experimentally as has been discussed previously. Platelets will rapidly adhere and aggregate if the endothelial barrier is breached as has been shown in many studies in experimental animals (reviewed by Mustard *et al*, 1983) and the platelets adherent to collagen release the contents of their alpha and dense granules. Fissuring of an atherosclerotic plaque would also expose collagen fibres and the adhering platelets could release

agents such as thromboxane A_2 and ADP which would rapidly lead to adhesion and aggregation of more platelets and growth of the thrombus. In addition prostacyclin generation is probably reduced in the region of the atherosclerotic plaque possibly due to the high content of lipid peroxides in advanced atherosclerotic lesions (Glavind *et al*, 1952) as it is known that fatty acid peroxides inhibit prostacyclin generation (Moncada *et al*, 1976; Salmon *et al*, 1978). Thus Larrue *et al* (1980) demonstrated that the production of prostacyclin by cultured smooth muscle cells obtained from atherosclerotic plaques of the experimental atherosclerotic rabbit consistently produced less prostacyclin when compared to control cells. Furthermore prostacyclin production measured by bioassay and 6-oxo prostaglandin $F_{1\alpha}$ concentrations was reduced in perfused heart preparations from atherosclerotic rabbits (Dembinska-Kiec *et al*, 1977). Similarly prostacyclin production in human atherosclerotic tissue appears to be reduced. Sinzinger *et al* (1979) demonstrated decreased prostacyclin generation from atherosclerotic tissue compared to normal arterial tissue and could find no difference between early and advanced lesions. D'Angelo *et al* (1978) found no prostacyclin generation in atheromatous plaques from 3 subjects. In addition to the probable decreased prostacyclin production in the region of the atheromatous plaque possibly due to inhibition of prostacyclin synthetase by lipid peroxides it is known that certain fatty acid hydroperoxides also induce platelet aggregation (Mickel and Horbar, 1974). Thus diminution of prostacyclin production and stimulation of platelet aggregation both mediated by lipid peroxides could contribute to thrombosis on atheromatous plaques.

Increased thromboxane A_2 production may also contribute to thrombogenesis in atherosclerotic vessels. Thus platelet thromboxane A_2 production (measured as the stable degradation form thromboxane B_2) is increased in

patients with arterial or venous thrombosis (Lagarde and Dechavanne, 1977) and myocardial infarction (Szczeklik *et al*, 1978). Similarly in animal models, arterial blood thromboxane B_2 levels were increased in rabbits made atherosclerotic by cholesterol feeding (Shinamoto *et al*, 1978) and following acute ligation of the coronary artery in dog, high levels of thromboxane B_2 were found to be associated with cardiac arrhythmias (Coker *et al*, 1981). In addition thromboxane B_2 levels were found to be elevated in the peripheral blood of patients with Prinzmetal angina (Lewy *et al*, 1979; Robertson *et al*, 1981) and in coronary sinus blood of patients with unstable angina (Hirsch *et al*, 1981).

Although the above description of experimental findings would suggest that exposure of collagen and lipid peroxides due to rupture of the atheromatous plaque could be of importance in thrombogenesis together with enhanced platelet thromboxane A_2 formation and diminished vessel wall prostacyclin formation, Born (1983) has questioned this series of events. He has pointed out that platelet aggregates grow very rapidly and, in contrast, although platelet adhesion to collagen is almost instantaneous there is a lag period (several seconds) before aggregation proceeds (Wilner *et al*, 1969). In addition mural platelet thrombi can occur during passage of anticoagulated blood through artificial extracorporeal systems (Richardson *et al*, 1976) which suggests that under certain circumstances collagen or other vessel wall constituents are unnecessary for activation of platelets. Born (1983) has assembled evidence to suggest that the important trigger to *in vivo* thrombogenesis is ADP and that at sites of vascular injury enough ADP is released from damaged cells to initiate thrombogenesis (Born, 1983).

Whatever the physiological stimulus to platelet aggregation and thrombus

formation it appears that it is the fissuring of an atheromatous plaque often with extrusion of plaque lipid contents which triggers thrombus formation (Bouch and Montgomery, 1970). The nature of this obviously very important event remains unclear. However a possible explanation has been put forward by Brooke *et al* (1971) which proposes that softening of the atheromatous plaque may be due to the presence of unusual lipids (cholesteryl esters of hydroxyoctadecadienoic acid) found in high concentrations in ulcerated plaques (Harland *et al*, 1971) which are thought to produce smooth muscle cell necrosis at the base of the plaque.

Atherogenesis

As has been discussed above platelets are a crucial factor in thrombogenesis associated with atheromatous arteries. However over recent years the possible role of platelets in the early stages of the atheromatous process has been emphasized (Ross and Glomset, 1976). Of course the advanced atherosclerotic plaque is a very complex structure (Woolf, 1983) but the early lesion is very much a process which involves the intima of the artery and is characterised by proliferation of smooth muscle cells which have migrated from the media of the artery. The fact that this early lesion is intimal suggests a response to a factor or factors in the blood and the "response to injury" hypothesis is central to current thinking on the atherosclerotic process. It was Virchow in the middle of the nineteenth century who first put forward the concept of arterial wall injury leading to the development of the atheromatous plaque. In the middle of the twentieth century Duguid (1946) emphasized the importance of arterial thrombi in the genesis of the plaque and pointed out that it was Rokitansky (1844) who first put forward the 'encrustation hypothesis'. The role of platelets in the process (which has resulted from the work of

several groups, French, 1966; Chandler and Hand, 1961; Murphy *et al*, 1962 and Ross *et al*, 1974) as put forward by Ross and Glomset (1976) is really a bringing together of the Virchow and Rokitansky hypotheses of more than a century ago in that platelets may themselves contribute to vessel injury, thrombosis and atherogenesis (Mustard *et al*, 1983).

The functions of the endothelial cells of the arterial intima have been reviewed by Majno and Joris (1978) who highlighted several important aspects of endothelial cell physiology including a barrier function to the formed elements of blood and plasma macromolecules; resistance to platelet thrombi; active transport of substances from blood; synthesis of factors involved in vessel wall/blood interactions and vascular repair processes. Thus endothelial cells synthesize many elements of their underlying connective tissue including fibronectin, elastin, collagen, proteoglycans and microfibrils (Bornstein and Sage, 1980; Buonassisi, 1973; Howard *et al*, 1976 and Jaffe *et al*, 1976) which controls vessel wall permeability as well as stimulating thrombosis following loss or damage to endothelial cells (Smith *et al*, 1979). Exchange of macromolecules across the endothelium involves active vesicle formation, and transendothelial channels and intercellular clefts (Chien, 1978; Simionescu *et al*, 1976). The resistance of intact endothelium to platelet aggregation does not solely depend on its ability to synthesize prostacyclin as has been discussed previously. Other factors which may be involved in this important property are secretion of plasminogen activator (Loskutoff and Edgington, 1977); a membrane associated ADPase (Lieberman *et al*, 1977); the presence of heparin-like membrane proteoglycans (Thorgeirsson and Robertson, 1978); the ability to take up and degrade vasoactive amines (Johnson and Erdos, 1977); the uptake and clearance of circulating thrombin (Lollar and Owen, 1980) and surface charge (Sawyer and Srinivasan, 1973).

From the above description it can be seen that the endothelial cell has many important properties and endothelial damage is proposed as the initial event in the 'response to injury' hypothesis. In the postulated series of events breach of the endothelial barrier would expose sub-endothelial smooth muscle cells and connective tissue to plasma constituents including lipoproteins, platelets and macrophage-type mononuclear cells (Harker and Ross, 1979). Following endothelial injury, platelets would adhere to the injured site, form microthrombi and release their granular contents which include a potent alpha-granule derived mitogen that stimulates migration and focal proliferation of intimal and medial smooth muscle cells (Ross and Vogel, 1978). Subsequently there would be synthesis of collagen, elastin and proteoglycans by smooth muscle cells, intracellular and extracellular lipid accumulation and thrombosis associated with the lesion. Support for this sequence of events has come from animal models of atherosclerosis and studies using cell culture techniques and as Born (1983) has pointed out caution is necessary in translating these findings to the human situation. However there does seem to be considerable evidence linking platelets to the development of proliferative arterial intimal lesions following experimental removal of endothelium in animal experiments either mechanically or chemically (reviewed by Mustard *et al*, 1983). In addition the emerging information regarding the properties of the platelet-derived growth factor argues for an important role in the atherogenic process of this platelet alpha-granule constituent.

Platelet-derived growth factor has been highly purified and appears to consist of two polypeptide chains of molecular weight 14000-17000 covalently joined by disulphide bonds (reviewed by Bowen-Pope and Ross, 1984). Following its purification it has been possible to study the biological properties of platelet-derived growth factor. Important

amongst these properties are its ability to induce chemotaxis by vascular smooth muscle cells (Grotendorst *et al*, 1982; fibroblasts (Seppä *et al*, 1982) and monocytes (Deuel *et al*, 1982); to increase rates of fluid phase pinocytosis (Davies and Ross, 1978) and protein synthesis (Owen *et al*, 1982); to increase the number of LDL receptors (Chait *et al*, 1980) and somatomedin receptors (Clemmons *et al*, 1980) as well as to stimulate proliferation of connective tissue cells. It remains to be seen whether cells can respond to platelet-derived growth factor *in vivo* and at the moment its role in atherogenesis remains speculative but attractive.

As has been discussed the 'response to injury' hypothesis depends on initial endothelial injury and little is known about loss of endothelium *in vivo* either spontaneously or in response to risk factors for atherosclerosis. Although endothelial replication is increased in response to hypertension or hyperlipidaemia (Florentin *et al*, 1969; Schwartz *et al*, 1980) this does not necessarily imply that the arterial intima is denuded of endothelium. In addition electron microscopic studies have not shown significant areas of denuded intima in early hyperlipidaemia in animal models despite there being increased endothelial turnover (Bondjers *et al*, 1977 and Taylor *et al*, 1978). These findings have led to the suggestion that altered endothelial function without endothelial loss may be important in permitting platelet thrombus formation. In this regard Gryglewski *et al* (1978) showed that prostacyclin formation in segments of blood vessel from cholesterol-fed monkeys was reduced. These authors, on the basis of these experiments, suggested a primary role for prostacyclin deficiency in the development of atherosclerosis in these animals. However this suggestion depends on the assumption that prostacyclin is continually produced by arterial wall to prevent platelet aggregation. This assumption is not tenable as it is now recognised that the unstimulated

output of prostacyclin by blood vessels is low (reviewed by Dollery *et al*, 1983). However factors that reduce the ability of the vascular endothelium to produce prostacyclin may impair the ability of the vessel to response to vascular injury (Dollery *et al*, 1983).

Recent work from Faggiotto *et al* (1984) and Faggiotto and Ross (1984) has cast more light on the 'response to injury' hypothesis in cholesterol fed monkeys. These workers have emphasized that the initial lesion in this model of atherogenesis is the adherence to endothelium of monocytes which then appear to migrate into the sub-endothelium, accumulate lipid and become lipid-laden macrophages or foam cells. It was only after some months that endothelial denudation was observed with platelet mural thrombus attached to exposed lipid containing macrophages. These authors postulate that in as yet an unexplained way it is the accumulation of these lipid-laden foam cells in the sub-endothelium that damage the endothelium thus allowing the subsequent developments in the formation of the atheromatous plaque to proceed.

Platelets and Diabetes Mellitus

Introduction

It has been seen that platelets may have not only a crucial role in thrombogenesis but also in the development of the atheromatous plaque. This has stimulated interest in the possible role of these factors in the premature vascular disease both atherosclerosis and microangiopathy, seen in subjects with diabetes mellitus. In addition platelet aggregates have been observed in some diabetic patients with microangiopathy and in experimental animals (Bloodworth *et al*, 1965) and microvascular thrombi strongly suggestive of platelet aggregates have been observed in sural nerve biopsies of diabetic patients (Timperly *et al*, 1975) and in patients

dying from diabetic ketoacidosis (Timperly *et al*, 1974). More recently thrombi consisting primarily of platelets and fibrin have been observed in the retinal microvessels of the experimental rat some months after the induction of diabetes (Ishibashi *et al*, 1981).

However despite the increasing numbers of research communications concerned with platelet function in the diabetic there remain many conflicting reports and unresolved questions. Many factors contribute to this unsatisfactory situation. For instance there is no absolutely satisfactory test of platelet function. Many studies have been performed using *in vitro* tests and it is difficult to be certain as to the relevance of these tests to platelet function *in vivo*. Platelets are studied in an artificial environment and the very process of preparing the platelet sample for study may affect the population of platelets studied and their behaviour. Despite this there is no doubt that the development of the turbidometric method for the study of platelet aggregation *in vitro* (Born, 1962) gave tremendous impetus to the study of platelet function. In more recent years as understanding of platelet physiology has increased newer tests have been applied to the study of platelets from diabetic subjects which may be more relevant to platelet function *in vivo*. Two of these developments, the measurement of platelet-specific proteins as measures of *in vivo* platelet activation and release reaction and the impedance aggregometer which allows the assessment of platelet aggregation in whole blood have been used in this thesis. For convenience these tests together with the results of other studies using these tests will be discussed in the Discussion section of this thesis.

In recent years the importance of the prostaglandin pathway in platelets in relation to platelet aggregation has become apparent and several studies have attempted to assess the activity of this pathway in platelets from diabetic subjects. In addition the effect of the diabetic state on

prostacyclin synthesis and the balance between prostacyclin and thromboxane has been examined. For convenience, studies of the platelet prostaglandin pathway, prostacyclin and thromboxane A_2 in diabetes will be described along with the results obtained in this thesis in the Discussion section.

Further attempts to estimate platelet function *in vivo* have involved isotopic labelling of platelets and measurement of platelet survival. However these tests also have potential limitations, the study of large patient groups is not practical and the results must be interpreted with caution.

In addition to problems intrinsic to the methodology of platelet function measurement problems also arise from the diabetic groups studied. Many of the early studies of platelet function in diabetic subjects failed to take into account other factors known to influence platelet function, particularly the presene or absence of diabetic vascular complications. This has led to difficulties in interpretation of the possible sequence of events in the development of enhanced platelet reactivity in diabetics. In other words are observed platelet abnormalities related to the disturbed metabolic state of diabetes or are they secondary to the presence of vascular disease? More recent studies have attempted to overcome this problem by electively studying diabetics free from clinical evidence of vascular disease. However it is difficult to be completely sure of the absence of vascular disease on clinical grounds alone and for this reason some groups have studied platelet function in diabetic children and adolescents in whom vascular disease would be unlikely.

Platelet Adhesion Studies

Early studies of platelet function in diabetes used *in vitro* tests of platelet adhesion. A variety of techniques were used involving glass beads or fibres in a column. Anticoagulated blood or PRP

was passed through the column at a fixed flow rate and platelet counts were performed before and after exposure to these foreign surfaces - the difference being the number of platelets adhering. In general these techniques are tricky and difficult to reproduce and conflicting results were reported. Whereas increased platelet adhesion was reported in diabetics with evidence of macro- and microvascular disease (Mayne *et al*, 1970) no increase in adhesion was found in diabetics with retinopathy (Heath *et al*, 1971). However the majority of studies did show increased adhesiveness in diabetic subjects with (Baldwin *et al*, 1970; Valdorf-Hansen, 1967; Hellem, 1971) and without (Hellem, 1971; Shaw *et al*, 1967) vascular disease.

Platelet Aggregation

For *in vitro* aggregation studies citrated blood is carefully centrifuged to produce PRP. Aliquots of PRP are then placed in an aggregometer and stirred at 37°C. Various agonists, e.g. ADP, collagen, adrenaline and thrombin are then added to the PRP to induce aggregation which is measured as a change in optical density.

Studies of *in vitro* platelet aggregation in diabetics date from the late sixties and early seventies. These early studies were performed in diabetics with and without clinical evidence of vascular disease. However as Colwell *et al* (1983) have pointed out, if enhanced platelet aggregation were to contribute to the pathogenesis of diabetic vascular disease then evidence of it should be present before the development of vascular disease. Later studies have taken care to define the diabetic population studied in terms of the presence or absence of complications. Increased platelet aggregation has been reported in patients with neuropathy (O'Malley *et al*, 1975), retinopathy (Heath *et al*, 1971; Bensoussan *et al*, 1975) and coronary artery disease (Szirtes, 1970). However conflicting findings have appeared. For instance in studies of retinopathy patients

- the marker most commonly used of microvascular disease - patients exhibited enhanced platelet aggregation to the agonists adrenaline and arachidonic acid but not to ADP in a report of 25 patients (Creter *et al*, 1978). On the other hand platelet sensitivity to ADP was exaggerated in retinopathy patients studied by Khosla *et al* (1979) and was significantly greater than that in diabetics without retinopathy.

The majority of reports of platelet aggregation studies in diabetics without clinical evidence of vascular complications have found increased aggregation to various agonists particularly ADP and collagen (Sagel *et al*, 1975; Halushka *et al*, 1977; Stuart *et al*, 1979; Silberbauer *et al*, 1981; Halushka *et al*, 1981; Janka *et al*, 1981). However the occasional study has failed to demonstrate increased platelet aggregation in diabetics free of vascular disease (Petersen *et al*, 1978; Corbella *et al*, 1979) but certainly it would appear from the studies as a whole that platelet aggregation is more enhanced in diabetics with complications.

Spontaneous Platelet Aggregation

Several studies have attempted to assess whether there is evidence of increased circulating platelet aggregates in diabetic subjects. The platelet aggregate ratio as described by Wu and Hoak (1974) depends on the ratio of the platelet count performed on PRP derived from blood mixed with formalin to that in PRP prepared without formalin. Formalin fixes platelet aggregates which would be removed during the centrifugation to produce PRP. Therefore the platelet aggregate ratio would be low in the presence of circulating platelet aggregates.

It is important to remember that other factors may affect the platelet aggregate ratio such as the rate at which blood is drawn through the sampling needle (Rohrer *et al*, 1978). However using this method Davis *et*

al (1982) demonstrated that a group of 15 insulin dependent diabetics without clinical evidence of complications had a significantly reduced platelet aggregate ratio of 0.75 ± 0.6 (± SD) compared to 0.91 ± 0.11 in age- and sex-matched control subjects. No difference in the platelet aggregate ratio was found between a group of 15 diabetics diagnosed after the age of 30 years (6 treated with insulin), some of whom had vascular complications compared to age- and sex-matched controls. No details were given of the glycaemic control or other metabolic parameters of the two groups of diabetics studied (Davis *et al*, 1982) and it is difficult to account for these findings. Dettori *et al* (1983) assessed the platelet aggregate ratio in 92 'adult onset' diabetics and compared the results to 50 control subjects of similar age. No differences were observed in the diabetic groups as a whole compared to controls. However when the patients were divided into those with good control (glycosylated haemoglobin < 9%) and bad control (glycosylated haemoglobin > 9%) it was found that the group with poor control had a significantly reduced platelet aggregate ratio. Along with this there was a highly significant inverse relationship between platelet aggregate ratio and glycosylated haemoglobin. No such correlation was seen with various parameters of lipid metabolism (Dettori *et al*, 1983). In a further study of the platelet aggregate ratio in diabetic subjects Preston *et al* (1978) found a significant reduction in the diabetic group and this was mainly due to those diabetics with evidence of microvascular complications.

The above studies have attempted to assess the presence of circulating platelet aggregates. Other studies have measured 'spontaneous' platelet aggregation in PRP and whole blood *in vitro* using a variety of techniques but mostly involving agitation by stirring. The platelet aggregates formed in this way are fixed with formol. It is difficult to assess the

significance of this 'spontaneous' platelet aggregation. However the phenomenon is unusual in healthy individuals and it is conceivable that circulation stresses may have similar effects on platelets *in vivo*. Studies of this kind have demonstrated increased 'spontaneous' *in vitro* platelet aggregation in insulin dependent diabetic subjects free from vascular disease (Paulsen *et al*, 1981; Krzywanek and Breddin, 1981; Silberbauer *et al*, 1981).

Platelet Survival

Reduced platelet counts have been reported in subjects with non-insulin dependent diabetes (Fuller *et al*, 1979) which could be due to increased platelet consumption. However there have been relatively few studies of platelet survival in diabetics and those that have been performed have involved small numbers. Eight insulin dependent diabetics were studied by Ferguson *et al* (1975) who measured platelet survival using *in vivo* labelling with [^{75}Se]-selenomethionine. Using this technique platelet survival was found to be reduced in the diabetic subjects and it was unlikely that the findings were due to pre-existing vascular disease as the subjects studied were reported to be free from vascular disease. This is of interest as in an earlier study using ^{51}chromium-labelled platelets Abrahamsen (1968) found reduced platelet survival only in diabetics with vascular complications. Dassin *et al* (1978) also used ^{51}chromium-labelled platelets to measure platelet survival in diabetics in a more recent study. In this study approximately a third of the diabetics studied appeared to have increased platelet turnover but this finding did not differ significantly from controls. In this study there did not appear to be any relationship between the presence of vascular complications and platelet survival (Dassin *et al*, 1978). A recent study

has attempted to assess the possible relationship between glycaemic control and ^{51}chromium platelet survival (Jones *et al*, 1981). These authors studied platelet survival in 12 diabetics during periods of hyperglycaemia and periods of euglycaemia and were unable to demonstrate any differences. Indeed platelet survival did not differ between diabetics and 6 control subjects overall. However 3 of the diabetics did show significantly reduced platelet survival and all 3 subjects had retinal disease suggesting that the platelet changes were secondary to the presence of vascular complications (Jones *et al*, 1981). The above studies obviously give somewhat conflicting results possibly related to the small numbers of diabetics studied and the different techniques used. However support for the findings of decreased platelet survival due to the diabetic state comes from a study in the experimental diabetic rat in which Johnson *et al* (1980) found decreased platelet survival.

Other workers have measured platelet lifespan by determining the recovery of platelet malondialdehyde formation (a measure of the prostaglandin pathway) following aspirin ingestion (Paton, 1979; Tindall *et al*, 1981). Paton (1979) used this technique to study 12 diabetics and 12 control subjects and the diabetics were found to have a faster return to normal of malondialdehyde formation suggesting more rapid platelet turnover. Similarly Tindall *et al* (1981) found a significantly reduced platelet lifespan in long-standing diabetics (duration > 10 years) whether or not microvascular complications were present.

Platelets and Lipids

In recent years increasing interest has been focussed on lipid platelet interactions (Editorial, 1980). It has been seen that platelet-derived growth factor has important effects on LDL receptor activity (Chait *et al*, 1980) and it is known that platelets which are unable to synthesize

cholesterol *de novo* possess specific LDL receptors (Aviram *et al*, 1980). In addition lipoproteins have important actions on prostacyclin production. Thus LDL appears to inhibit the production of prostacyclin by arterial wall as does VLDL whereas HDL stimulates prostacyclin production (Norday *et al*, 1978). In addition animal studies in which atherosclerosis has been induced by cholesterol feeding have emphasized concurrent changes in platelets. Thus Wu *et al* (1975) noted increased platelet aggregates 6 weeks after feeding an atherogenic diet containing 1.2% cholesterol to rhesus monkeys. In addition to these findings early studies of platelets from hyprcholesterolaemic patients demonstrated enhanced platelet aggregation (Carvalho *et al*, 1974). For convenience this and other studies of platelet function in hyperlipidaemic states will be discussed along with the findings reported in this thesis in the Discussion section.

AIMS

The aims of the studies to be reported in this thesis are to apply newer and possibly more physiological tests of platelet function to clinically and biochemically well-documented diabetic and hyperlipidaemic subjects who are known to be at risk from premature vascular disease.

1. The platelet-specific proteins β-thromboglobulin and platelet factor 4 will be measured in platelet-poor plasma in patients with diabetes mellitus and age- and sex-matched non-diabetic control subjects. The diabetics will be carefully examined for the presence or absence (as far as possible) of large and small vessel vascular disease so that the levels of platelet-specific proteins can be assessed in diabetics with and without complications. In addition glycaemic control will be determined in the diabetics by measurement of glycosylated haemoglobin levels and fasting individual lipid and lipoprotein concentrations will be performed. Correlations will be sought between platelet-specific protein levels and these measures of the metabolic abnormalities in the diabetics.

2. The platelet-specific proteins will be measured in a group of clinically and biochemically well-documented hyperlipidaemic subjects compared to age- and sex-matched controls. The different types of hyperlipidaemic subjects as described in the introduction will be assessed individually. As patients with hyperlipidaemia are more likely to have large vessel disease (which may be undetected clinically) an additional control group of patients with known peripheral vascular disease will be studied.

3. Platelet malondialdehyde production in washed platelets stimulated with arachidonic acid will be studied as a measure of the activity

of the platelet prostaglandin pathway in subjects with diabetes and hyperlipidaemia and compared to control subjects.

4. The sensitivity of platelets from diabetic subjects to the anti-aggregatory effects of prostacyclin will be studied *in vitro* and compared to the responses seen in non-diabetic healthy controls.

5. The production of prostacyclin by tissues from 'acutely' and 'chronically' experimental diabetic pregnant rats will be measured to determine the time course of the known reduction in aortic prostacyclin production and to see whether prostacyclin production in another tissue, namely myometrium, is similarly affected.

6. Platelet aggregation will be studied in whole blood samples from diabetic subjects using the new impedenace aggregometer (Cardinal and Flower, 1980). This may be a more physiological approach to the study of platelet aggregation.

METHODS

Measurement of Triglyceride and Cholesterol Concentrations

Total serum triglyceride and triglyceride concentrations of individual lipoprotein preparations were measured by a semi-automated fluorometric method (Cramp and Robertson, 1968).

Total serum cholesterol and cholesterol concentrations of lipoprotein preparations were measured using Lieberman-Burchard reagent with a Locarte fluorimeter.

Isolation of Individual Lipoproteins

Individual fasting lipoprotein levels were measured by precipitation techniques.

VLDL was precipitated from 1 ml of serum in 17 x 58 mm glass tubes by the addition of 75 µl of 10% (W/V) SDS (BDH Chemicals Ltd.) in 0.15 mol/l sodium chloride (Ononogbu and Lewis, 1976). After mixing, the tubes were incubated at 37°C in a water bath for 2 h. The tubes were then centrifuged (in an angle-head rotor) at 10,000 x g for 20 min (in an MSE high speed 18 centrifuge). The clear infranatant was removed without disturbing the floating VLDL precipitate. The tube but not the VLDL pellet was washed with 0.1% SDS (W/V) in 0.15 mol/l sodium chloride. The VLDL pellet was then redissolved in 1 ml of 1% (W/V) SDS in 0.15 mol/l sodium chloride at 37°C and an aliquot extracted in isopropanol for measurement of VLDL triglyceride and VLDL cholesterol. This method has been reported to give results which correlate well with VLDL measurements obtained by ultracentrifugation. For VLDL cholesterol, Ononogbu and Lewis (1976) found a correlation coefficient, $r = 0.98$ and for VLDL triglyceride, $r = 0.99$. In a series of 15 fasting serum samples obtained from patients studied for this thesis analysed by this method and by ultracentrifugation

(Havel *et al*, 1955) correlation coefficients for VLDL triglyceride and VLDL cholesterol of r = 0.93 and r = 0.92 were obtained.

Chylomicra, VLDL and LDL were precipitated from serum by precipitation with polyanions (Burstein *et al*, 1970). Heparin (250 units in 50 µl) (Weddel Pharmaceuticals) and manganese chloride (50 µl of 1 mol/l solution) were added to serum (1 ml) in glass tubes on ice and the contents mixed on a vortex mixer. The tubes were left for 15 min on ice to allow complete precipitation of apoprotein B containing lipoproteins and then centrifuged at 2000 x g for 20 min at 4°C. An aliquot of the resulting supernatant was then taken for measurement of HDL cholesterol and HDL triglyceride.

LDL cholesterol and LDL triglyceride values were obtained by subtracting the sum of HDL and VLDL values from the total serum cholesterol and triglyceride concentrations. In a series of 15 fasting serum samples obtained from patients studied for this thesis in which HDL concentrations were measured by heparin/manganese precipitation and by ultracentrifugation correlation coefficients were obtained for HDL cholesterol and HDL triglyceride of 0.93 and 0.91 respectively.

Platelet-Specific Proteins

Blood sampling for platelet-specific protein estimations were performed using a large-bore needle and without venous occlusion. Blood samples were immediately transferred to the sampling tubes (supplied with the assay kits) which had been pre-cooled on a mixture of ice and water. The samples were mixed with the anticoagulant by gentle inversion of the tubes and replaced immediately in the ice/water mixture for 15 min. Samples were then centrifuged at 2000 x g at 4°C for 30 min to obtain platelet-poor plasma (PPP). Following centrifugation the top 0.5 ml PPP was carefully removed, and stored at -20°C prior to assay.

β-Thromboglobulin and platelet factor 4 were measured by radioimmunoassay (β-thromboglobulin, βTG RIA kit, Amersham and platelet factor 4, PF4 RIA kit, Abbott Laboratories, U.K.). These methods depend on competition between non-radioactive β-thromboglobulin (or platelet factor 4) and ^{125}I-labelled β-thromboglobulin (or platelet factor 4) for a limited number of binding sites on specific antibodies to the platelet-specific protein. Thus, the amount of radioactive β-thromboglobulin (or platelet factor 4) bound to antibody will be inversely proportional to the concentration of non-radioactive β-thromboglobulin (or platelet factor 4) in the specimen. Antibody bound β-thromboglobulin or platelet factor 4, both radioactive and non-radioactive, is separated by precipitation with ammonium sulphate solution, the radioactivity in this complex being measured using a gamma scintillation counter (Denley Automatic Gamma Counting System, Searle Analytic Inc.).

The concentration of β-thromboglobulin (or platelet factor 4) in the plasma sample was determined by comparison with a group of standards containing known amounts of non-radioactive β-thromboglobulin (or platelet factor 4). For both assays all estimations were performed in duplicate and the results were expressed as the means of the duplicate assays. For the β-thromboglobulin assay the within assay coefficient of variation was 6.9% and the between assay coefficient of variation was 10.4%. For the platelet factor 4 assay the within assay coefficient of variation was 7.7% and the between assay coefficient of variation was 6.3%. Samples from hyperlipoproteinaemic subjects were routinely measured undiluted and diluted 1:2 and 1:4 to detect any non-linearity of the assay due to high lipid concentrations.

Malondialdehyde Formation by Platelets

Platelet malondialdehyde formation used for the assessment of prostaglandin synthesis was performed in washed platelets stimulated by

arachidonic acid (de Haas *et al*, 1979). PRP was obtained following centrifugation of citrated (3.8%) venous blood at 170 x g for 15 min at room temperature. Platelet concentration in the PRP was determined with a Coulter Thrombo-Counter Model C. ADP (2 µmol)(Sigma) was added to the tubes containing PRP and the tubes inverted several times to obtain flocculent aggregates (Mohamed *et al*, 1975). The tubes were then centrifuged at 170 x g for 2 min to obtain a platelet pellet. The supernatant was discarded and the pellet resuspended in 10 ml of modified Tyrode's buffer containing sodium chloride 137 mmol/l, potassium chloride 2.68 mmol/l, calcium chloride 1.8 mmol/l, glucose 5.5 mmol/l, sodium dihydrogen orthophosphate ($NaH_2PO_4 2H_2O$) 0.32 mmol/l and magnesium chloride ($MgCl_2 6H_2O$) 2 mmol/l.

Bovine serum albumin (0.35%) W/V)(Sigma) and potato apyrase (0.01% W/V) (Sigma) were added to this platelet suspension and the tubes incubated for 15 min at 37°C. Following incubation the platelet suspension was centrifuged at 490 x g for 15 min at room temperature and resuspended in buffer aliquots of 10^9 platelets/ml. Arachidonic acid (final concentration 100 µmol/l) emulsified in physiological saline was added to the platelet suspension and the mixture incubated for 15 min at 37°C. The reaction was then stopped by the addition of 1 ml of thiobarbituric acid (36.7 mmol/l) in perchloric acid (229 mmol/l)(Stuart *et al*, 1975). The tubes were then centrifuged at 2500 x g for 20 min at 4°C. The supernatant was decanted, boiled for 20 min and then allowed to cool at room temperature. The volume was then made up to 3 ml and the pink chromagen read at 532 nm using a 1 cm light path (Flower *et al*, 1973). Results were expressed in nmol/10^9 platelets using an extinction coefficient of 1.37×10^5 for a molar solution.

Measurement of Glycosylated Haemoglobin

Glycosylated haemoglobin which is synthesized slowly over the life span of erythrocytes provides an objective means of assessing the average blood glucose concentration present in the weeks before sampling (Bunn *et al*, 1976; Koenig *et al*, 1976). The percentage of glycosylated haemoglobin was estimated using a microscale ion-exchange column method (Welch and Boucher, 1978) for the study on platelet-specific proteins and malondialdehyde formation. For the study on whole blood platelet aggregation in insulin-dependent diabetics, glycosylated haemoglobin was measured using agar gel electrophoresis (Corning).

Prostacyclin Production in Rat Tissue

Induction of Diabetes

Female Wistar rats (200-250 g) were made diabetic by injection of streptozotocin (80 mg/kg)(Sigma) into the tail vein. Streptozotocin 2 deoxy-2-(3-methyl-3-nitrosoureido) D-glucopyranose, produces diabetes in rats following a single injection (Rakietin *et al*, 1963). Its diabetogenicity is related to pancreatic islet β-cell cytotoxicity through the nitrosourea moiety. Control rats received an injection of vehicle only. The induction of diabetes was confirmed by blood glucose measurements using a glucose oxidase method; blood glucose concentration ranged from 15-20 mmol/l in the diabetic animals. One group of animals was made diabetic and mated after 5 weeks - the chronically diabetic group. A further group of animals was mated and diabetes was induced between days 7-17 of pregnancy (acutely diabetic). Animals were sacrificed on days 20-22 of pregnancy (day 22 = day of delivery).

Tissue Isolation and Prostacyclin Estimation

Aortic and myometrial tissue samples were prepared as described by Williams and El Tahir (1980a) and suspended (25% W/V) in gassed Kreb's

solution, pH 8.0.

The reaction was initiated by chopping the sample finely with subsequent incubation for 15 min at 20°C. Aliquots of incubation media were then removed and stored on ice prior to assay.

The antiaggregatory activity in samples of incubation media was estimated against authentic prostacyclin (a gift of Wellcome Research) using rabbit citrated PRP. This technique is highly selective for prostacyclin. Estimates in myometrial incubation media show close correlation with 6-oxo-prostaglandin$_{1\alpha}$ levels determined by gas-chromatography/mass spectrometry and the intra- and inter-assay coefficients of variation were 5.6% and 6.9% respectively (Williams and El Tahir, 1980b).

Platelet Sensitivity to Prostacyclin

Venous blood was collected from overnight fasted control and diabetic subjects from an antecubital vein via a 19 gauge butterfly needle under gravity into 3.13% (W/V) sodium citrate (9 ml blood:1 ml citrate) contained in a plastic tube. The blood was centrifuged immediately at 200 x g for 10 min at room temperature to obtain PRP. Aliquots of PRP (0.5 ml) were placed in a Bryston aggregometer and stirred at 1100 revolutions/min at 37°C. Platelet aggregation induced by the addition of ADP (Sigma) produced changes in the optical density of the PRP which were measured on a chart recorder. In each experiment the minimal dose of ADP to produce irreversible aggregation for at least 3 min was determined. The ability of prostacyclin (a gift of Wellcome Research) to inhibit ADP-induced aggregation was investigated and a dose-response curve constructed. Prostacyclin was reconstituted in Tris buffer (0.1mol/l, pH 9.6) and added 1 min prior to ADP. From these data the dose of prostacyclin causing a 50% inhibition of ADP-induced aggregation (ID_{50}) was calculated.

Platelet Aggregation in Whole Blood

Venous blood was collected after an overnight fast from an antecubital vein via a 19 gauge butterfly needle under gravity into 3.13% (W/V) trisodium citrate, pH 7.4 (18 ml blood:2 ml citrate) in a plastic tube. Platelet aggregation was measured with a Chronolog Model 540 whole blood aggregometer (Coulter Electronics Ltd.) connected to a Rikadenki pen recorder. The aggregometer stirring speed was set at 600 rpm and temperature at 37°C. When the calibration button was depressed (giving a fixed impedence of 5 ohm), the gain control was adjusted such that a deflection of 25 mm was obtained. The recorder was set at 100 MV full scale deflection with a rate of 2 cm/min. Platelet aggregation was studied in response to two concentrations of collagen (1 µg/ml and 5 µg/ml) (Immuno Ltd.) and 1 mmol/l arachidonic acid (Sigma)(final conentrations). In addition the sensitivity of platelets to the antiaggregatory action of prostacyclin was assessed. Blood samples (adjusted to standardized haematocrit (0.300) with saline) (975 µl) were warmed for 3 min at 37°C in the machine heater block and then stirred with a teflon coated stir bar in a glass cuvette after inserting the impedance electrode. A period of 2 to 3 min was necessary for the platelet monolayer to form and the base line to become steady. The aggregating agent (25 µl) was then added and aggregation allowed to continue until a maximum was reached (up to 8 min). Both rate (Ω/min) and the extent of aggregation (Ω) were measured.

For the prostacyclin sensitivity studies, prostacyclin was added 1 min before the addition of arachidonic acid (final concentration 1 mmol/l). Prostacyclin was reconstituted in Tris buffer (0.1 mmol/l, pH 9.6) and 10 µl added to the whole blood sample (965 µl), final pH 7.4. Increasing concentrations of prostacyclin (0.5 - 20 ng/ml) were added and the mid-point between a concentration that did not inhibit and a concentration that inhibited totally is described here as the prostacyclin ID_{50}.

RESULTS

Studies in Diabetic Patients

Platelet-Specific Proteins

One hundred and forty-three diabetic subjects, 90 non-insulin dependent and 53 insulin dependent, were studied. All patients received a careful clinical examination with particular reference to the presence or absence of diabetic complications. Patients were examined for the presence of fundal microaneurysms, haemorrhages and exudates and new vessel formation following dilatation of the pupils. Forty-two patients had clinical evidence of retinopathy, twenty with new vessel formation. No patient had had recent laser therapy for retinopathy. The presence of large vessel disease was determined by the following criteria: previous myocardial infarction was recorded when electrocardiographic and cardiac enzyme evidence supported an appropriate clinical history. A positive history of angina pectoris was recorded when the patient experienced recurring attacks of typical exertional anterior chest pain, usually with radiation that was relieved by rest or nitroglycerin. A documented history or evidence of a previous cerebrovascular accident was recorded. Pain in the leg muscles when walking, especially in the calves, that disappeared with rest, was considered to be intermittent claudication. Loss of limb pulses was recorded. Twenty patients had clinical evidence of large vessel disease. None of the patients had evidence of renal impairment.

The diabetic patients were compared to groups of apparently healthy age- and sex-matched controls with no history and no clinical evidence of vascular disease drawn from laboratory and hospital staff. Both diabetics and controls had not taken platelet-suppressive drugs for at least two weeks prior to blood sampling. Diabetic patients had not suffered a hypoglycaemic reaction for at least four weeks prior to the study.

Plasma β-thromboglobulin was measured in all the one hundred and forty-three diabetic subjects and the results are shown in Figure 3. It can be seen that there is considerable overlap for β-thromboglobulin levels between diabetic and control subjects. However 27.3% of diabetics had β-thromboglobulin levels greater than the highest level seen in the control group. The mean and range of β-thromboglobulin levels for controls and diabetics are shown in Table 5.

Levels of platelet factor 4 found in diabetic and control subjects are shown in Figure 4 and Table 5. Platelet factor 4 was measured in 26 insulin dependent and 54 insulin independent diabetics (36 males and 44 females). 31.3% of diabetics had levels of platelet factor 4 greater than the highest level observed in age- and sex-matched controls.

Statistical analysis for both the β-thromboglobulin and platelet factor 4 results showed these differences to be highly significant (Table5).

No differences were found for β-thromboglobulin and platelet factor 4 levels between male and female diabetics or between insulin dependent and non-insulin dependent patients. There were strong correlations (Spearman test) between β-thromboglobulin and platelet factor 4 levels both in control subjects ($R = 0.5012$; $P < 0.001$) and diabetics ($R = 0.7631$; $P < 0.001$).

The platelet specific protein data in the diabetics were analysed in relation to the presence or absence of diabetic complications (Table 6). Those diabetics with clinical evidence of retinopathy had slightly higher levels of β-thromboglobulin and platelet factor 4 than those diabetics without retinopathy and these differences reached statistical significance for β-thromboglobulin (Table 6). Mean β-thromboglobulin (74.4 ng/ml) and platelet factor 4 (48.45 ng/ml) levels in diabetics with large vessel disease were not significantly higher than diabetics without vascular disease. However these diabetics together with those patients with no apparent vascular disease differed significantly from controls.

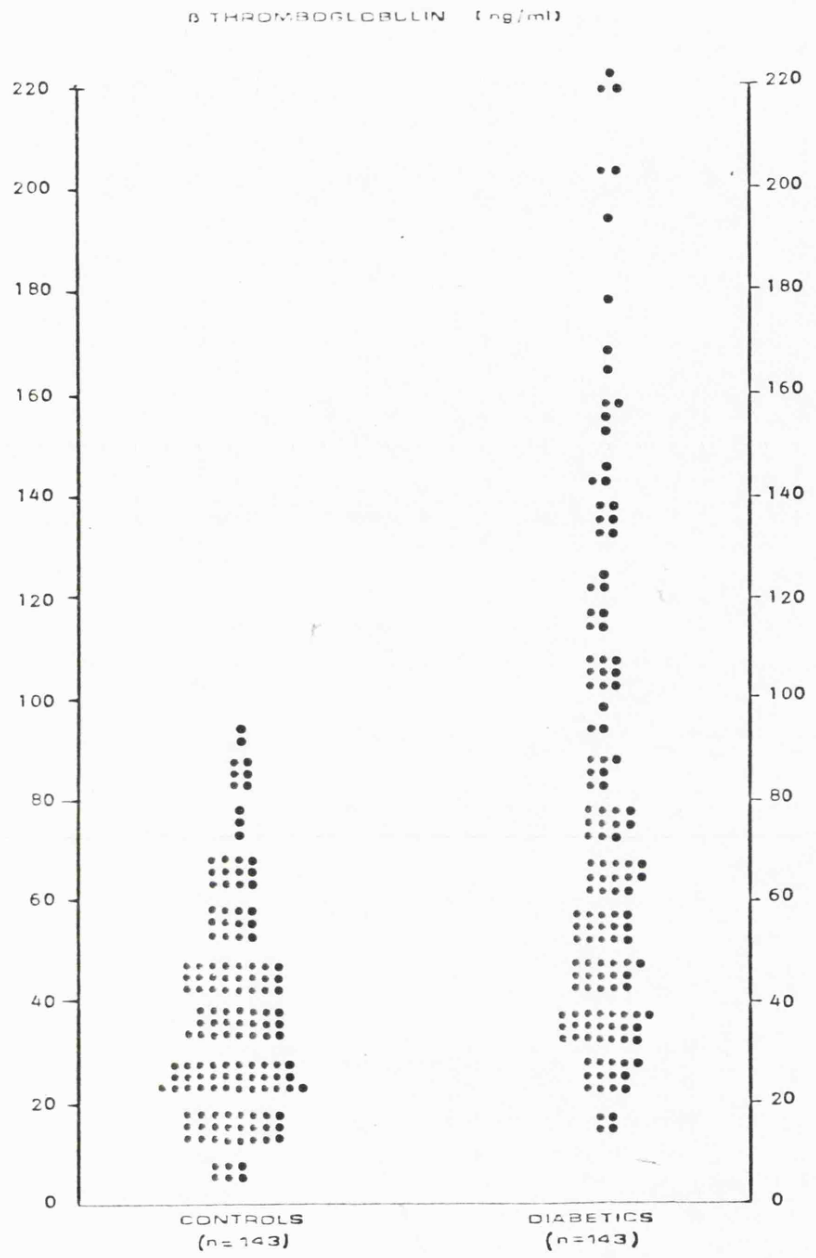

Figure 3

β-Thromboglobulin levels (ng/ml) in 143 diabetics and 143 age- and sex-matched controls. Points are means of duplicate assays.

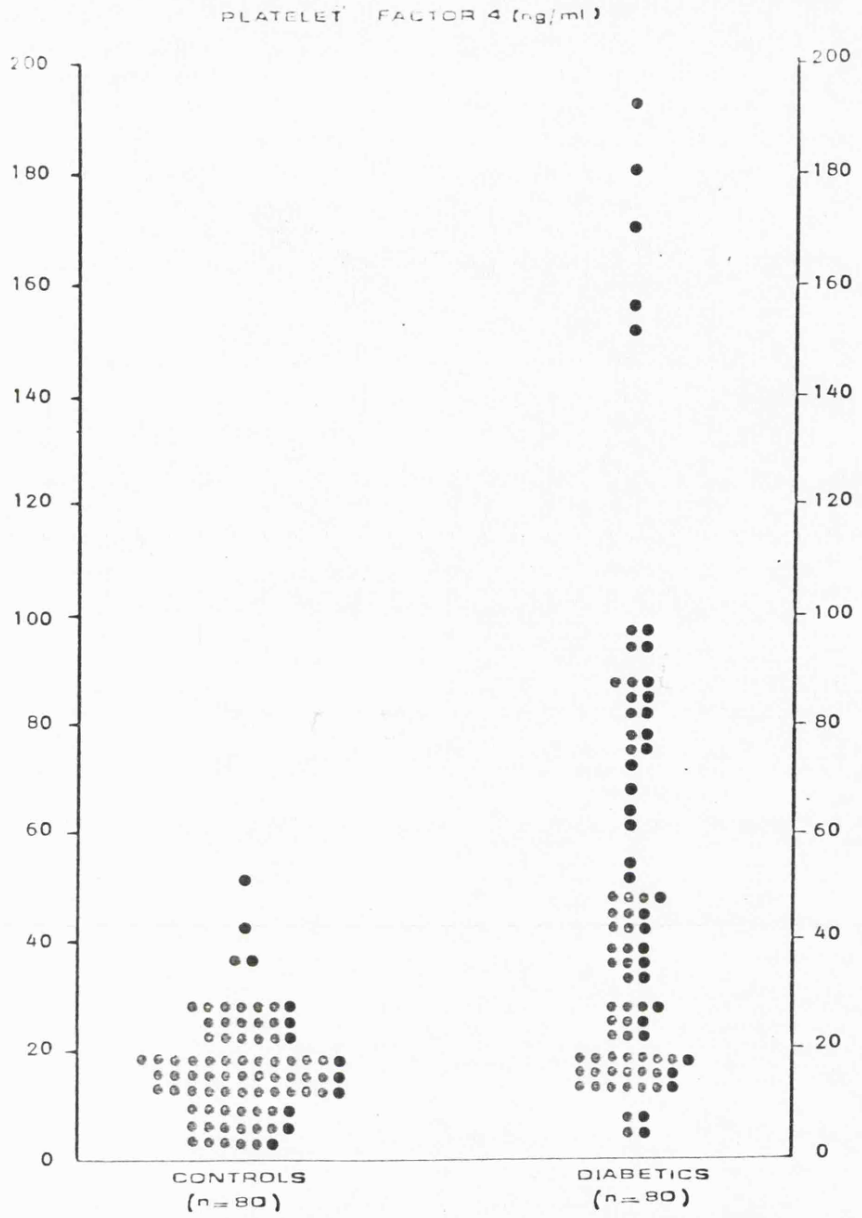

Figure 4

Platelet factor 4 levels (ng/ml) in eighty diabetics and eighty age- and sex-matched controls. Points are means of duplicate assays.

	CONTROLS			DIABETICS		
	Mean levels (range)	No.	Mean age (range)	Mean level (range)	No.	Mean age (range)
β-Thromboglobulin (ng/ml)	36.3 (8-95)	143	53.5 (21-80)	72.6* (14.0-220)	143	56.8 (20-81)
Platelet factor 4 (ng/ml)	16.5 (6.6-29.6)	80	55.9 (21-80)	48.5* (9.0-193)	80	57.8 (20-79)
Malondialdehyde formation ($nmol/10^9$ platelets)	8.1 (5.4-13.0)	30	29.7 (14-50)	12.4* (5.1-26.8)	48	53.7 (20-79)

* $P<0.0005$.

Table 5. Levels of plasma β-thromboglobulin, of platelet factor 4 and malondialdehyde formation in diabetic and control subjects. Statistical analysis by chi-squared test.

	Diabetic patients				
	With retinopathy	No.	Without retinopathy	No.	Significance
β-Thromboglobulin (ng/ml)	79.0 (18-167)	42	70.0 (14-220)	101	P<0.042
Platelet factor 4	55.9 (9.3-191.5)	32	44.3 (9.0-193)	48	NS

Table 6. Levels of plasma β-thromboglobulin and platelet factor 4 in diabetics with and without retinopathy. Statistical analysis by chi-squared test.

Table 7. Correlation between plasma β-thromboglobulin levels (βTG) and individual serum lipid and lipoprotein concentrations (Spearman correlation).

βTG *v.* Total triglyceride	R=0.3195	P≤0.029(n=49)
βTG *v.* VLDL triglyceride	R=0.3017	P≤0.041(n=48)
βTG *v.* Total cholesterol	N.S.(n=49)	
βTG *v.* LDL cholesterol	R=0.297	P≤0.042(n=49)
βTG *v.* HDL/Total cholesterol ratio (n=49)	R= -0.336	P≤0.02

In order to determine whether the elevated levels of platelet specific proteins observed in the diabetic group studied were related to diabetic control correlations were sought with glycosylated haemoglobin concentrations. Glycosylated haemoglobin levels were measured in 62 diabetic subjects (mean glycosylated haemoglobin 12.4%, range 8-19.6%). No correlation was found between levels of platelet specific proteins and the concentrations of glycosylated haemoglobin.

As important interactions have been described between lipoproteins and platelets and diabetes is an important cause of secondary lipid and lipoprotein abnormalities (see Introduction) correlations were sought with plasma levels of β-thromboglobulin and fasting plasma lipid and lipoprotein concentrations. These measurements were performed on 49 of the diabetic patients. Small but statistically significant correlations (Spearman test) were found between β-thromboglobulin levels and fasting plasma concentrations of individual lipids and lipoproteins as shown in Table 7.

Malondialdehyde Formation

Platelet malondialdehyde formation was measured *in vitro* in washed platelets stimulated by arachidonic acid in 23 insulin dependent and 23 non-insulin dependent subjects and compared to 30 control subjects (Table 5 and Figure 5). No difference in malondialdehyde formation was seen between platelets from non-insulin dependent and insulin dependent diabetic subjects.

34.1% of diabetics had levels of malondialdehyde formation greater than the highest result seen in the control group and this difference was highly significant (Table 5). Levels of malondialdehyde formation *in vitro* in diabetic subjects were significantly correlated (Spearman test) with plasma levels of β-thromboglobulin ($R = 0.4736$; $P < 0.002$, $n=46$) and with plasma levels of platelet factor 4 ($R = 0.6006$; $P < 0.009$; $n=21$).

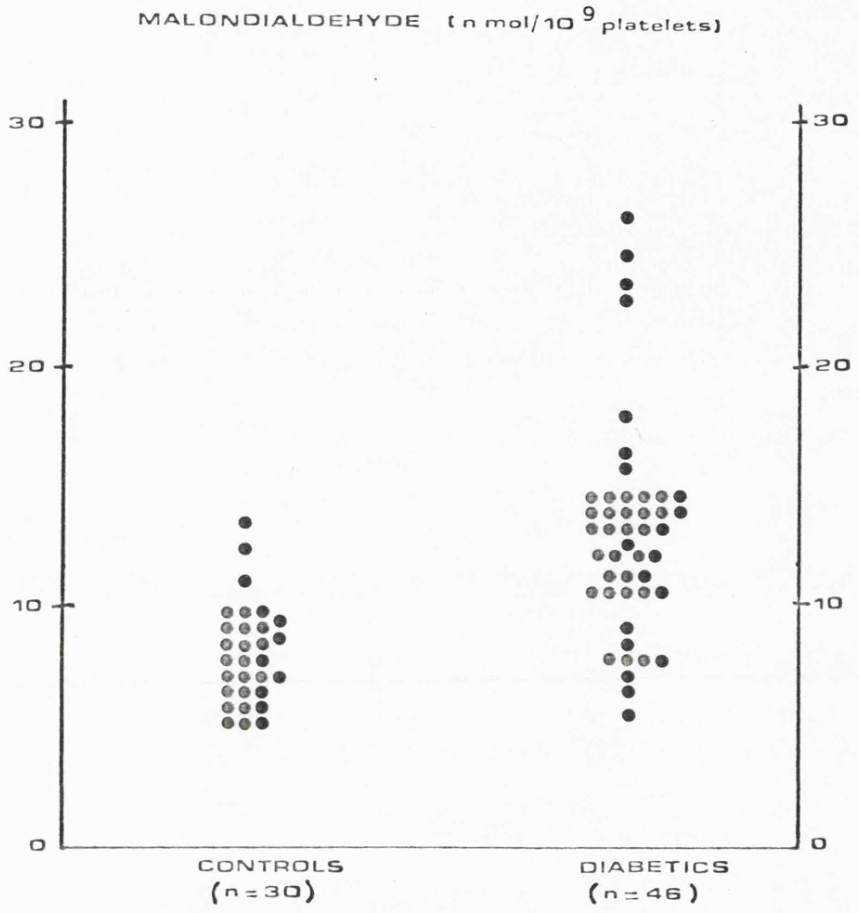

Figure 5

Malondialdehyde formation (nmol/10^9 platelets) in forty-six diabetics compared to thirty controls. Points are means of duplicate measurements.

Platelet Sensitivity to Prostacyclin

Sensitivity to the anti-aggregatory effects of prostacyclin was studied in a group of diabetic patients and control subjects. The clinical details of the diabetic patients studied are shown in Table 8. The presence or absence of complications in the diabetic patients was determined as previously described. The patients had had no recent hypoglycaemic attacks and mean preprandial blood glucose estimations ranged from 6.5 to 11.8 mmol/l. Control subjects were normal healthy volunteers from medical and laboratory staff. Neither diabetic patients nor control subjects had taken medication known to affect platelet function for at least two weeks prior to study.

An aggregation tracing using PRP from a control subject is shown in Figure 6a. Addition of ADP (final concentration 1 μmol/l) produced irreversible aggregation. Addition of prostacyclin to the PRP 1 min prior to the addition of ADP produced a dose-dependent inhibition of platelet aggregation. Figure 6b shows a similar aggregation tracing using PRP from a diabetic subject. Irreversible aggregation was produced by a similar concentration of ADP to that used in the control plasma. However, there was a marked difference in the concentration of prostacyclin required to inhibit ADP-induced aggregation. 1.25 ng prostacyclin added to the cuvette (final concentration 2.5 ng/ml) exerted minimal anti-aggregatory activity and addition of 5 ng (final concentration 10 ng/ml) was required to cause a 32% inhibition of the ADP-induced aggregation. This is 8 times the amount of prostacyclin needed to cause a similar inhibition to that in the control PRP.

Cumulative data from control and diabetic subjects obtained by the same experimental procedures are shown in Figure 7 and Table 8 As can be seen there is considerable overlap in prostacyclin ID_{50} values between diabetics and controls; however 30% of diabetics had ID_{50} values greater than the highest control value obtained. This difference was

Table 8. Clinical details and prostacyclin ID_{50} values (ng/ml) for diabetic patients and control subjects

	No.	Age (mean (range))	Sex	Duration (months) (mean (range))	prostacyclin ID50 (ng/ml) (mean (range))
Controls	34	34.5 (18-59)	15M, 19F		1.41 (0.5-3.8) (a)
Diabetics	40	59.3 (16-83)	19M, 21F	118.5 (1-428)	3.02 (0.48-11.2) (b)
IDDM	19	50.2 (16-82)	11M, 8F	123.2 (1-428)	2.81 (0.48-8.0) (c)
NIDDM	21	67.5 (46-83)	8M, 13F	152.1 (1-180)	3.2 (0.5-11.2) (d)
Diabetics with large vessel disease (15 IDDM, 5 NIDDM)	20	65.8 (54-82)	10M, 10F	103.5 (1-264)	3.87 (0.5-11.12) (e)
Diabetics without large vessel disease	20	52.7 (16-82)	9M, 11F	113.4 (1-428)	2.16 (0.48-6.4) (f)

(b) (c) (d) *v.* (a), $P<0.01$ (Wilcoxon Rank Sum Test); (e) *v.* (a), $P<0.002$; (e) *v.* (f), $P<0.01$; (c) *v.* (d) NS.

Insulin dependent diabetics - IDDM
Non-insulin dependent diabetics - NIDDM

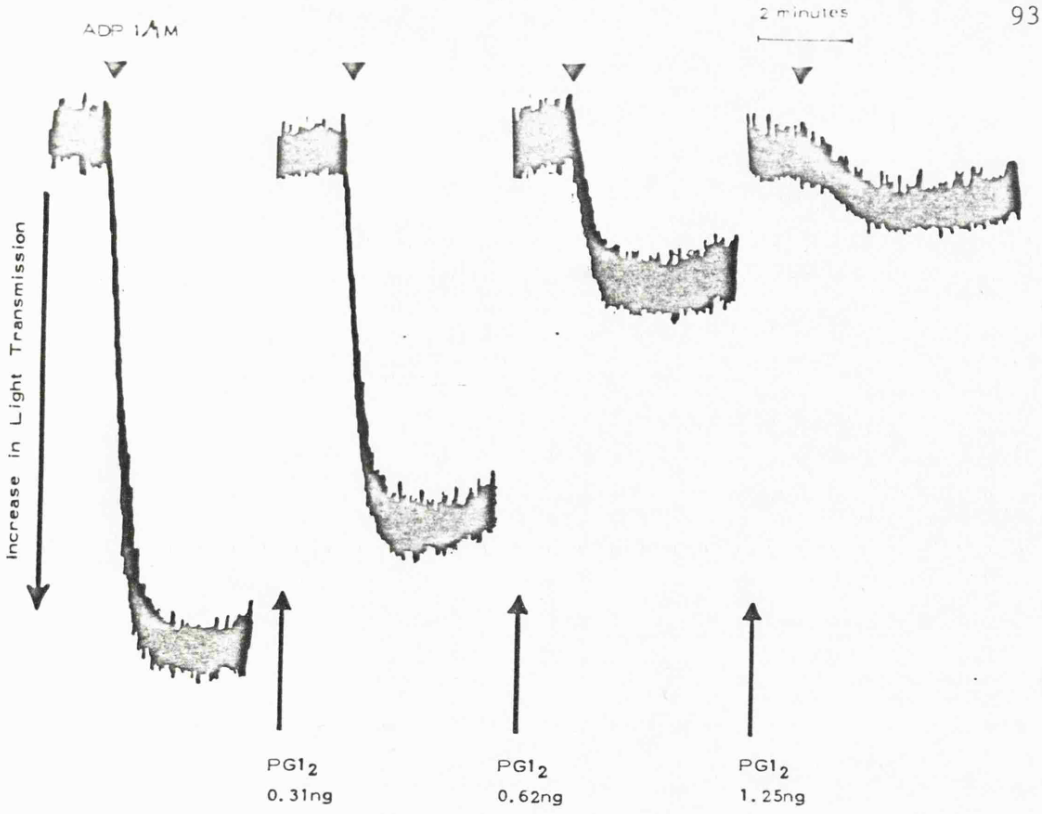

Fig. 6A

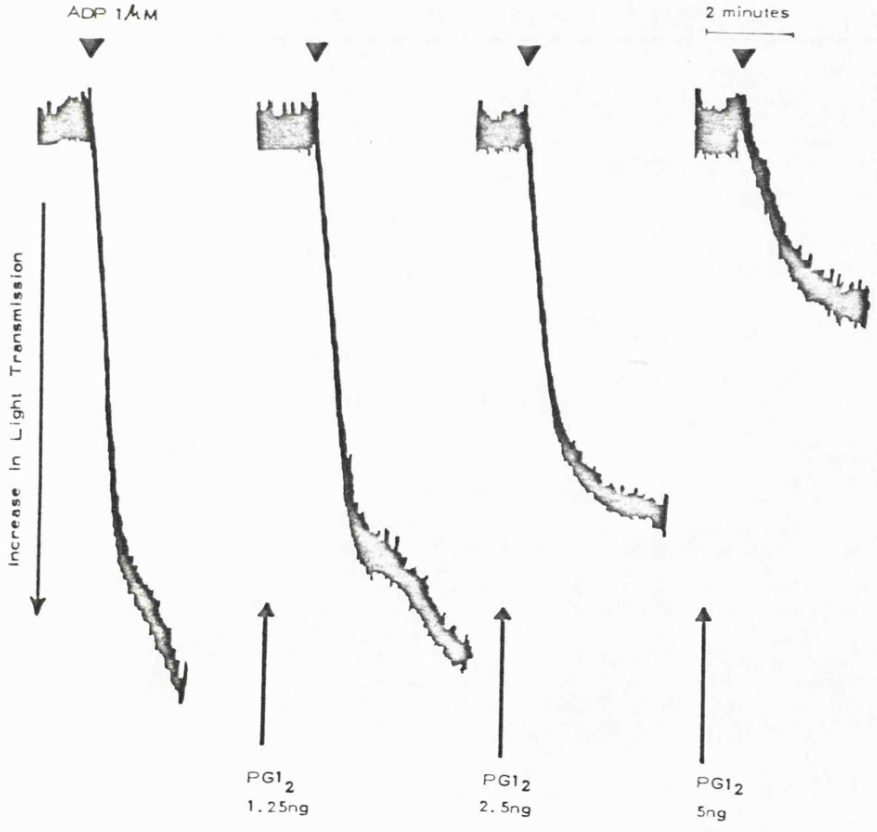

Fig. 6B

Platelet aggregation tracings showing the inhibitory effect of the prior addition of increasing amounts of prostacyclin on platelet aggregation induced by the addition of ADP; control subject (A) and diabetic patient (B). Prostacyclin = PGI_2

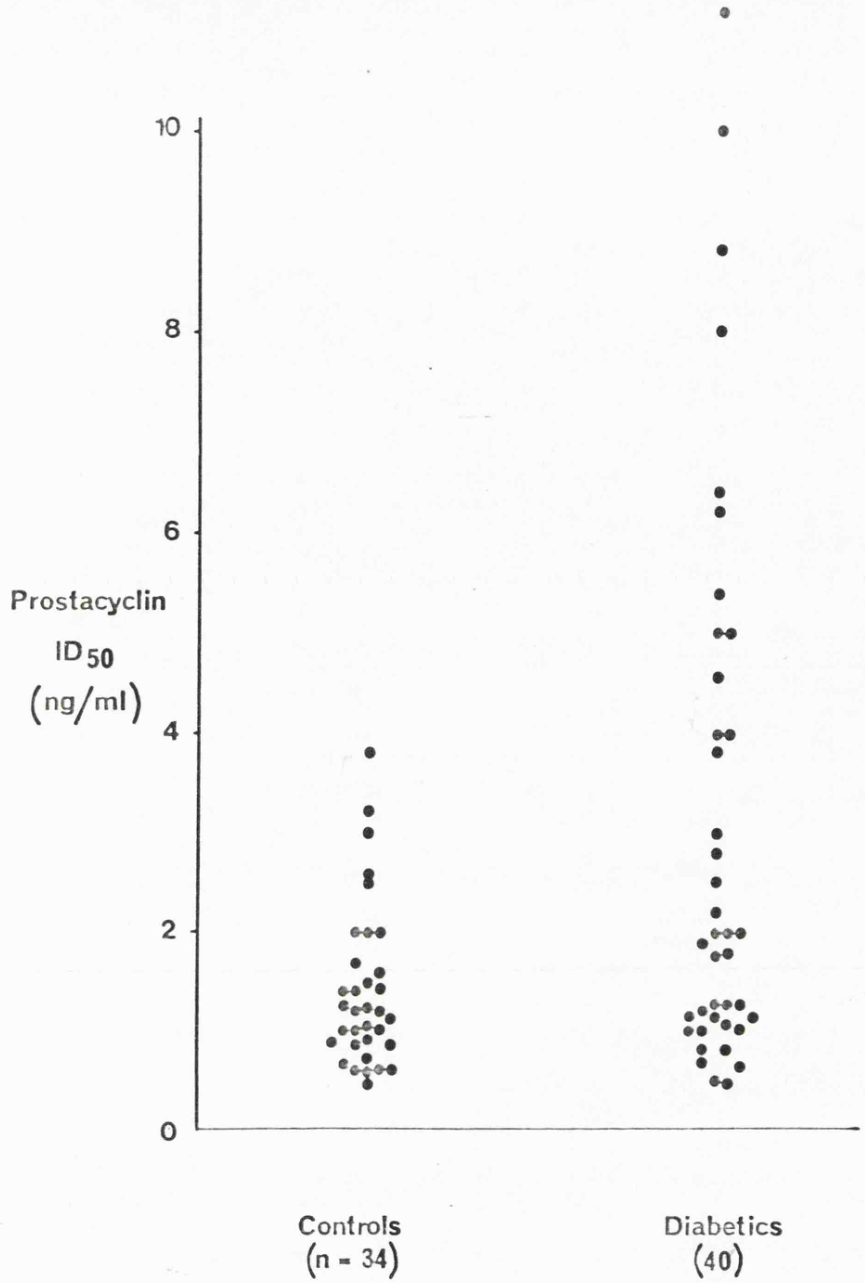

Figure 7

Calculated prostacyclin ID_{50} values (ng/ml) for platelets from control subjects and diabetic patients. $P<0.01$ (Wilcoxon Rank Sum test).

statistically significant using the Wilcoxon Rank Sum test, $P < 0.01$.

No difference was found in the sensitivity of platelets to prostacyclin between insulin dependent and non-insulin dependent patients (see Table 8) and there was no correlation with age in the control or diabetic group and no correlation with the duration of disease in the diabetics. Those diabetics with clinical evidence of large vessel disease had prostacyclin ID_{50} values much greater than those diabetics without vascular disease ($P < 0.01$). Diabetics without large vessel disease had higher ID_{50} values than controls but this difference did not reach statistical significance (see Table 8).

Prostacyclin ID_{50} values did not differ significantly between diabetics with retinopathy (n=9) and diabetics without retinopathy or control subjects.

Whole Blood Platelet Aggregation

Twenty insulin dependent diabetic subjects (12 females and 8 males, mean age 37.3 years, range 18-63 years) and 20 age- and sex-matched controls (mean age 38.6 years, range 24-58 years) were studied. The mean duration of diabetes was 12.8 years. Control subjects were normal healthy volunteers drawn from hospital and laboratory staff. No patients or control subjects had taken drugs affecting platelet function for at least twenty-one days and the diabetics had no recent history of hypoglycaemia. The diabetics chosen for this study had no clinical evidence of complications. Optic fundi were carefully examined following dilatation of the pupil to exclude retinopathy. The patients had normal renal function with no proteinuria and there was no clinical evidence of neuropathy. Clinical macrovascular disease was excluded as far as possible by a careful history and examination. Resting electrocardiograms were normal in all patients.

The results of whole blood platelet aggregation in response to collagen (1 µg/ml) and arachidonic acid(1mmol/l) for the diabetic and control groups are shown in Figure 8 and Figure 9 respectively.

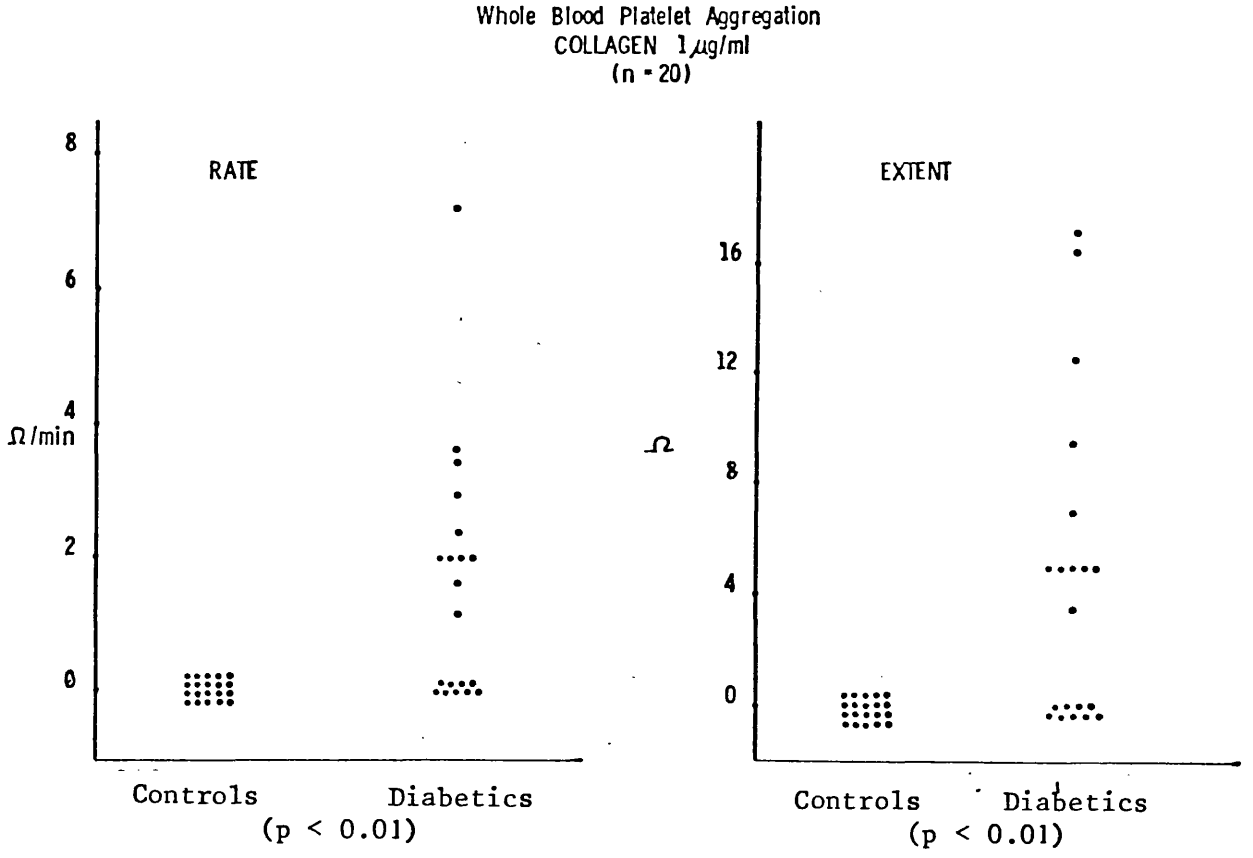

Figure 8

The rate (Ω/min) and the extent (Ω) of whole blood platelet aggregation induced by collagen (final concentration 1µg/ml) in diabetics and controls (n=20). Statistical analysis (Wilcoxon).

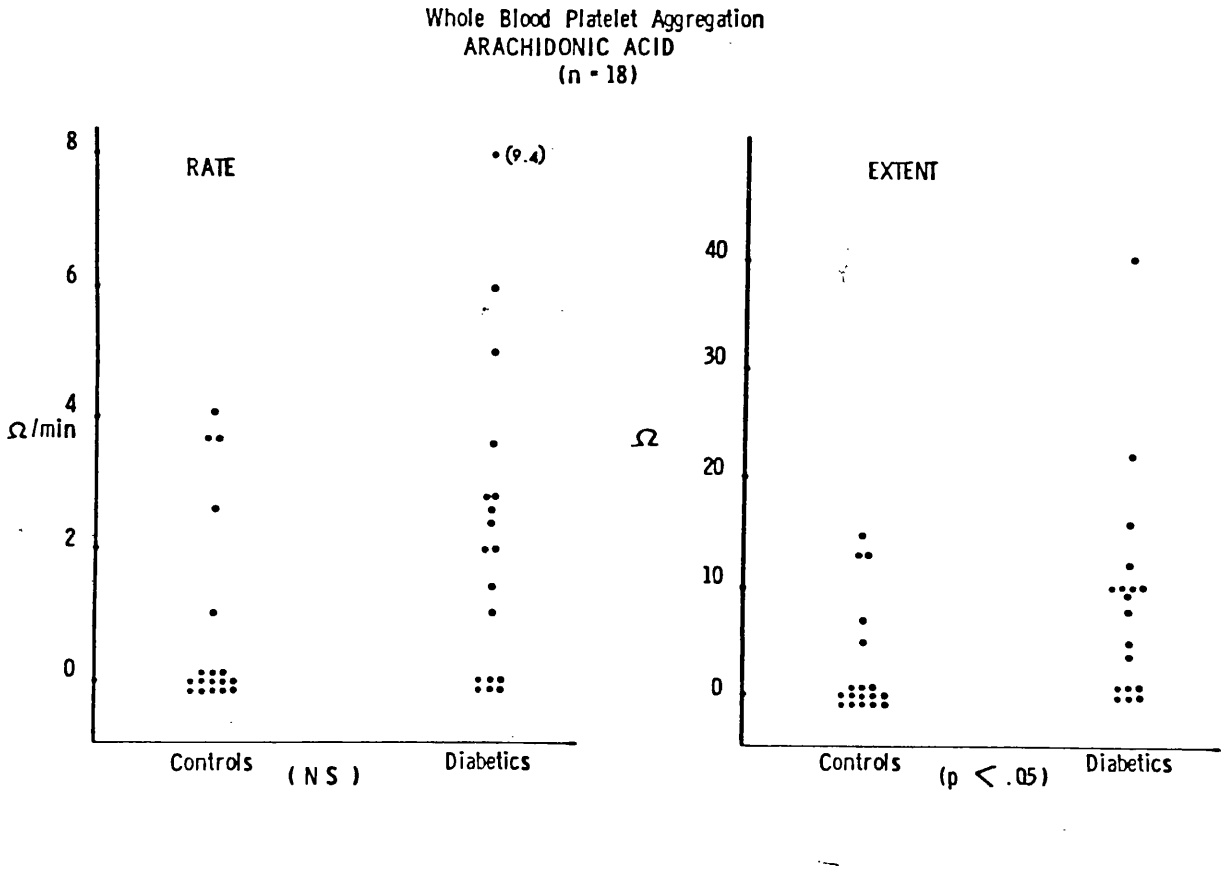

Figure 9

The rate (Ω/min) and the extent (Ω) of whole blood platelet aggregation induced by arachidonic acid (final concentration 1mmol/l) in diabetics and controls (n=18). Statistical analysis (Wilcoxon).

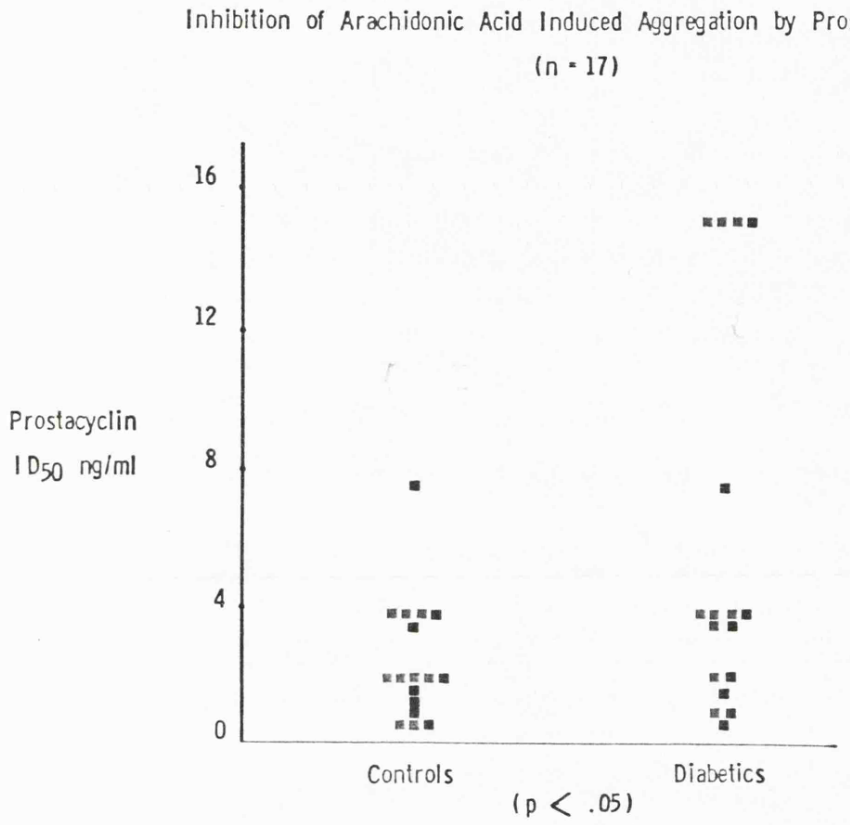

Figure 10
Calculated prostacyclin ID_{50} results (ng/ml) for diabetics and controls (n=17). ($p < 0.05$, Wilcoxon).

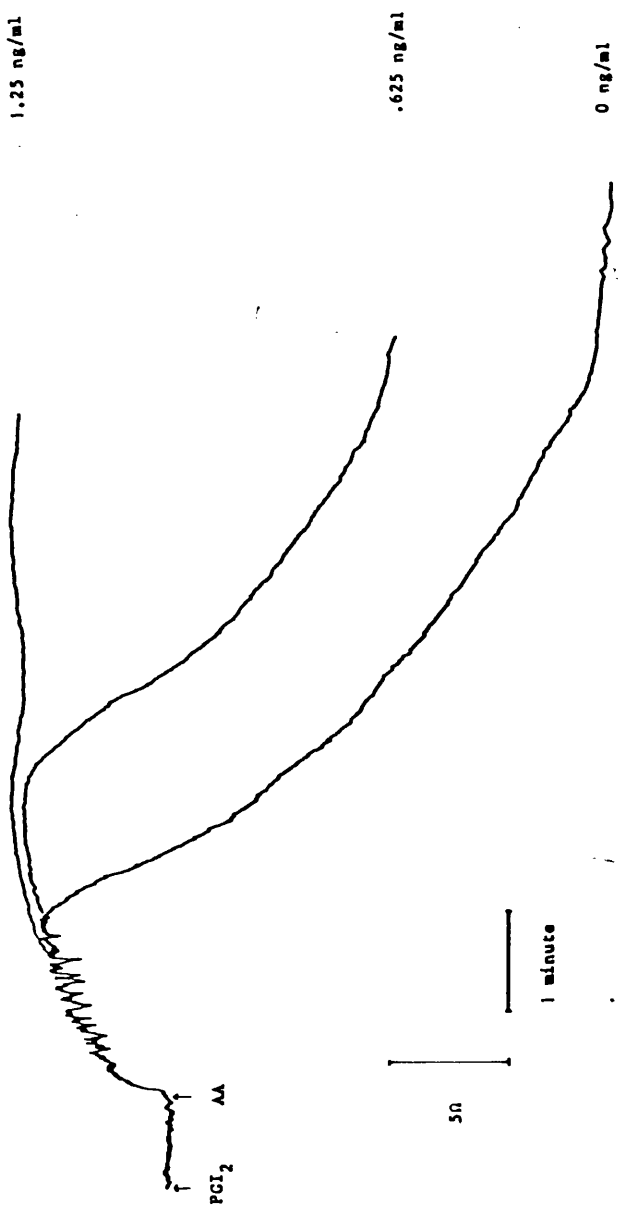

Fig. 11 A sample whole blood platelet aggregation tracing showing the inhibition of arachidonic acid induced platelet aggregation by increasing doses of prostacyclin.

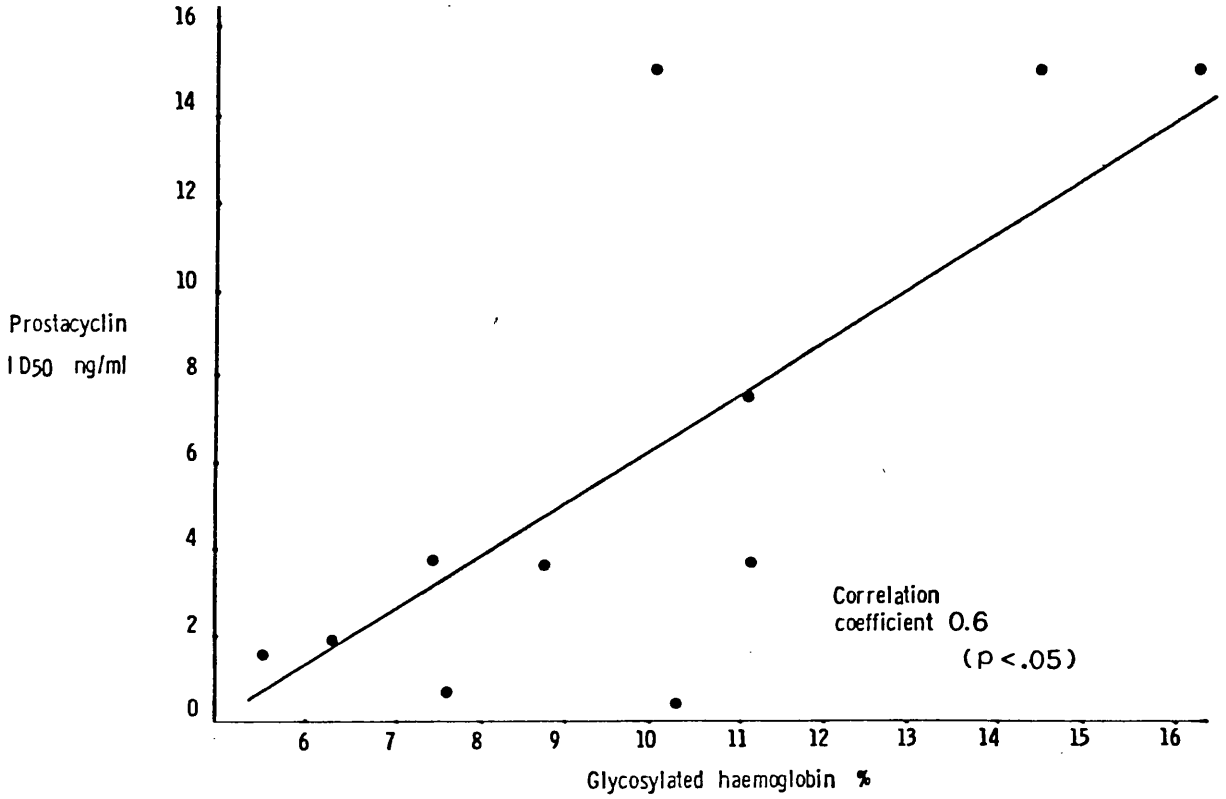

Figure 12

Correlation of prostacyclin ID_{50} results (ng/ml) for diabetics and the levels of glycosylated haemoglobin percentage.

(n = 11) ($p < 0.05$ Kendal).

It can be seen that both the rate and extent of aggregation to the collagen was significantly increased in the diabetic group compared to controls. A significant increase in the extent but not the rate of aggregation was seen for arachidonic acid. No significant difference was found between diabetics and controls in aggregation studies performed with collagen in higher dose, 5 µg/ml (data not shown).

The sensitivity of platelets in whole blood to the anti-aggregatory effects of prostacyclin is shown in Figure 10. A sample experimental tracing is shown in Figure 11. Diabetics showed a significantly reduced sensitivity to prostacyclin. In addition there was a significant positive correlation between glycosylated haemoglobin and the prostacyclin ID_{50} (Figure 12).

Prostacyclin Production by Tissues from Pregnant Diabetic Rats

The prostacyclin-like activity synthesised by tissues from acutely diabetic animals killed on day twenty-two of pregnancy is shown in Figure 13. Aortic tissue from control rats produced 5.75 ±1.23 ng prostacyclin/mg (mean ± SEM, n=8) and this was reduced to 3.45 ± 1.12 ng/mg in diabetic animals but this reduction was not statistically significant. Similarly there was no significant depression of myometrial prostacyclin production; control samples released 4.52 ± 0.3 ng/mg compared with 3.9 ± 0.31 ng/mg from the diabetic samples.

In chronically diabetic rats a marked reduction in aortic prostacyclin synthesis was noted; an aggregation tracing from one experiment is shown in Figure 14). The tracing shows the aggregation response induced in rabbit PRP by ADP (10 µmol/l final concentration). Equivalent volumes of myometrial incubation media from control and diabetic animals contained similar quantities of anti-aggregatory material. However an aliquot of aortic incubation medium from a control animal was found to contain 3 ng of prostacyclin whereas a similar volume of medium taken

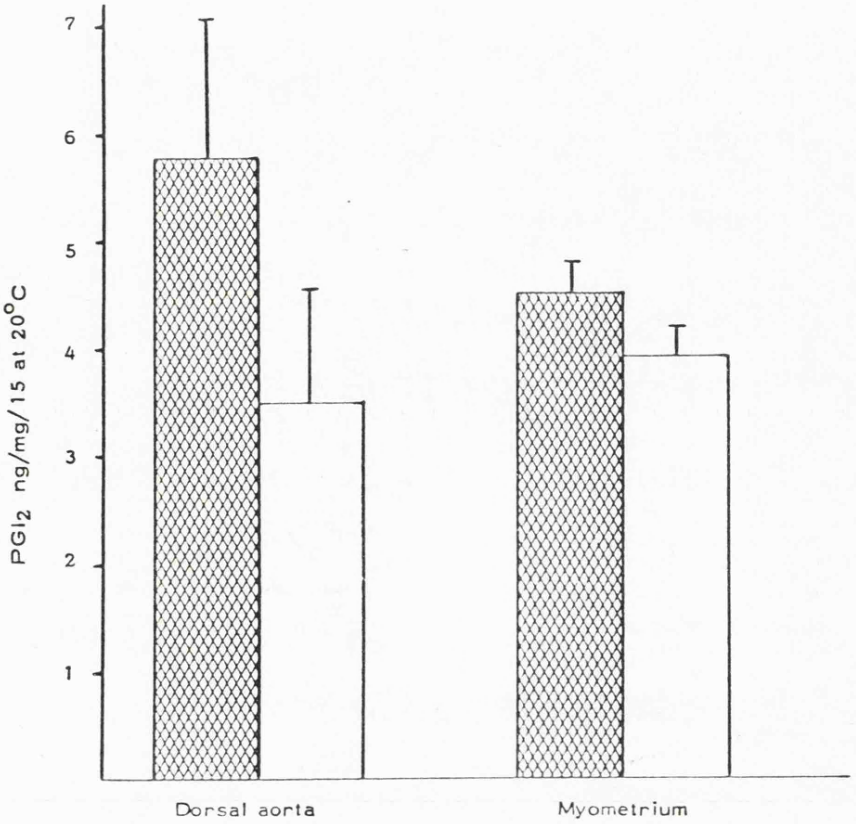

Figure 13

Effect of acute experimental diabetes on prostacyclin (PGI_2) production by aortic and myometrial tissues from pregnant rats. PGI_2 release from control (hatched bars) and diabetic tissues (open bars) are shown. Columns show mean ± SEM.

8 experiments. NS (Student's t-test).

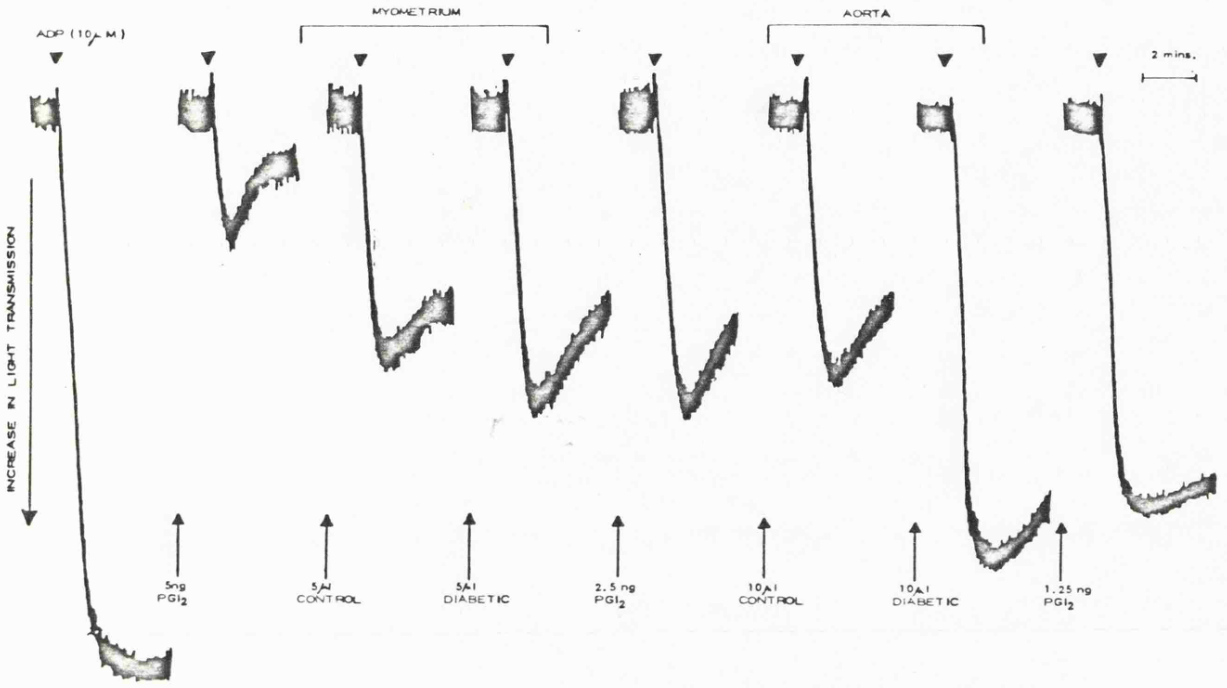

Figure 14

Aggregation tracing showing anti-aggregatory material released by aorta and myometrium from chronically diabetic rats. Aggregation was induced by ADP (10 μM). Authentic prostacyclin produced dose-related inhibitions of the ADP-induced response. Aliquots of myometrial incubation media from diabetic and control animals contained similar amounts of prostacyclin. However medium from the diabetic aortic sample contained markedly less prostacyclin than the control.

Prostacyclin = PGI_2

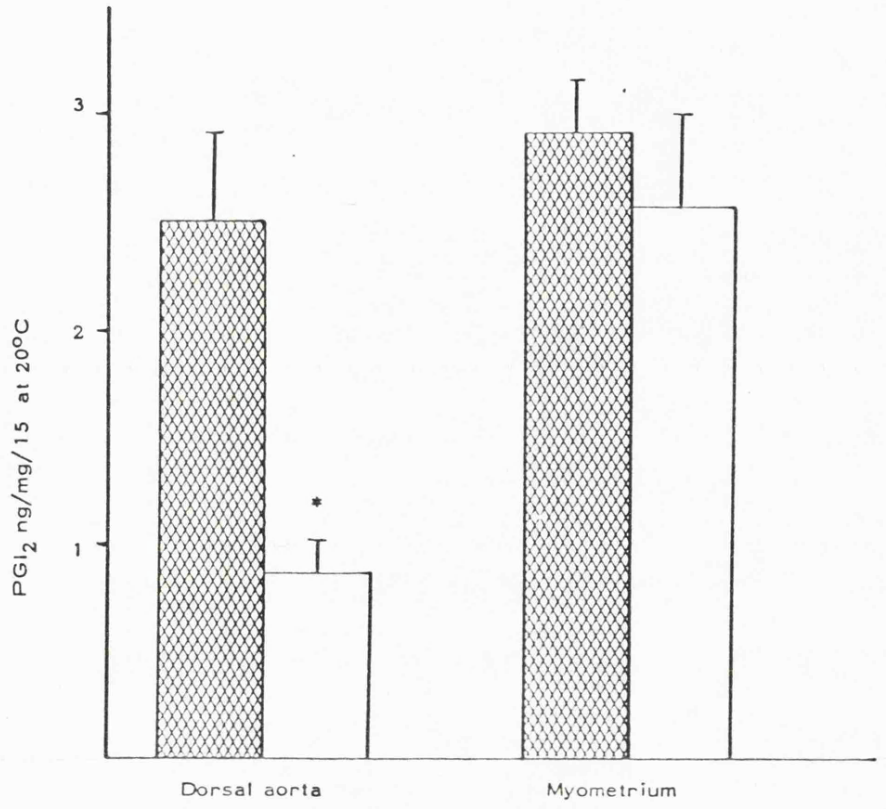

Figure 15

Effect of chronic experimental diabetes on prostacyclin (PGI_2) production by aortic and myometrial tissues from pregnant rats. Prostacyclin release from control (hatched bars) and diabetic (open bars) tissues are shown. Columns shown mean ± SEM.

9 experiments. *P<0.001. (Student's t-test).

after incubation of aorta from a chronically diabetic animal contained 1 ng. This represents a 60% decrease in prostacyclin release.

The cumulative findings from a series of experiments utilizing chronically diabetic animals are shown in Figure 15. In aortic tissue control prostacyclin synthesis was 2.5 ± 0.39 ng/mg compared with 0.85 ± 0.12 ng/mg in tissue from chronically diabetic rats, this reduction is significant ($P < 0.001$). However prostacyclin output by myometrial tissue from control and diabetic animals did not differ.

Studies in Hyperlipidaemic Patients

Platelet Specific Proteins

Sixty-nine patients with hyperlipidaemia were studied and compared to age- and sex-matched healthy controls drawn from hospital and laboratory staff (Table 9). The hyperlipidaemic subjects were classified according to the World Health Organisation classification (Beaumont *et al*, 1970). None of the patients had diabetes mellitus, thyroid disease, hepatic or renal disease. None of the hyperlipidaemic women was taking the contraceptive pill. In addition to the hyperlipidaemic and control groups, 57 patients (mean age 63.5 ± 2.11 SEM) with peripheral vascular disease and no diabetes or hyperlipidaemia (cholesterol < 7 mmol/l; triglyceride < 2.0 mmol/l) were studied by measurement of β-thromboglobulin values in PPP. The patients with peripheral vascular disease were clinically well documented by the Department of Vascular Surgery, King's College Hospital.

The mean plasma β-thromboglobulin was higher in the hyperlipidaemic patients than in the healthy controls (Figure 16). There was some overlap between the β-thromboglobulin values of patients and controls but in 46 patients (21 with type IIa, nine with IIb and sixteen with type IV), or 67% , the values were beyond the highest value for the control group.

Hyperlipidemia	No. and Sex	Age (yr)*	
		Patients	Controls
IIa	18, M	50.3 ± 2.54	48.8 ± 4.58
	14, F	53.4 ± 2.88	52.1 ± 3.62
IIb	13, M	53.7 ± 3.04	54.8 ± 4.6
	2, F	46.5	47
IV	18, M	50.5 ± 2.04	49.2 ± 4.2
	4, F	57.5 ± 1.66	56.5 ± 2.4
Total	49, M	51.3 ± 1.43	50.5 ± 2.79
	20, F	53.5 ± 2.43	52.5 ± 2.84

* Values given are the mean ± 1 standard error of the mean.

Table 9 Clinical details of hyperlipidemic patients classified according to the WHO classification and age- and sex-matched healthy controls in this study.

M male
F female

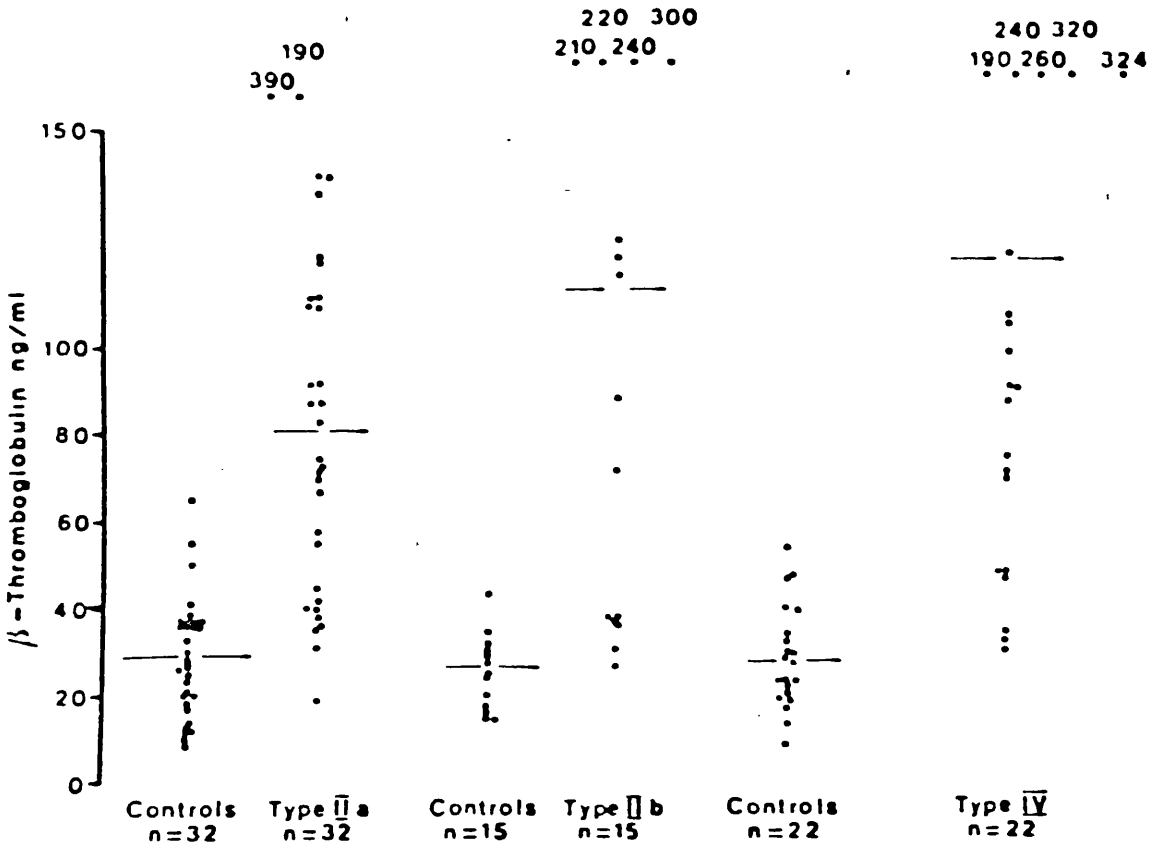

Figure 16

Mean (horizontal lines) and range of plasma β-thromboglobulin (ng/ml) in 69 patients with three types of hyperlipidaemia, and age- and sex-matched controls. The difference (Student unpaired t-test) between the mean logarithmic values in patients with each type of hyperlipidaemia and controls was highly significant ($P<0.001$).

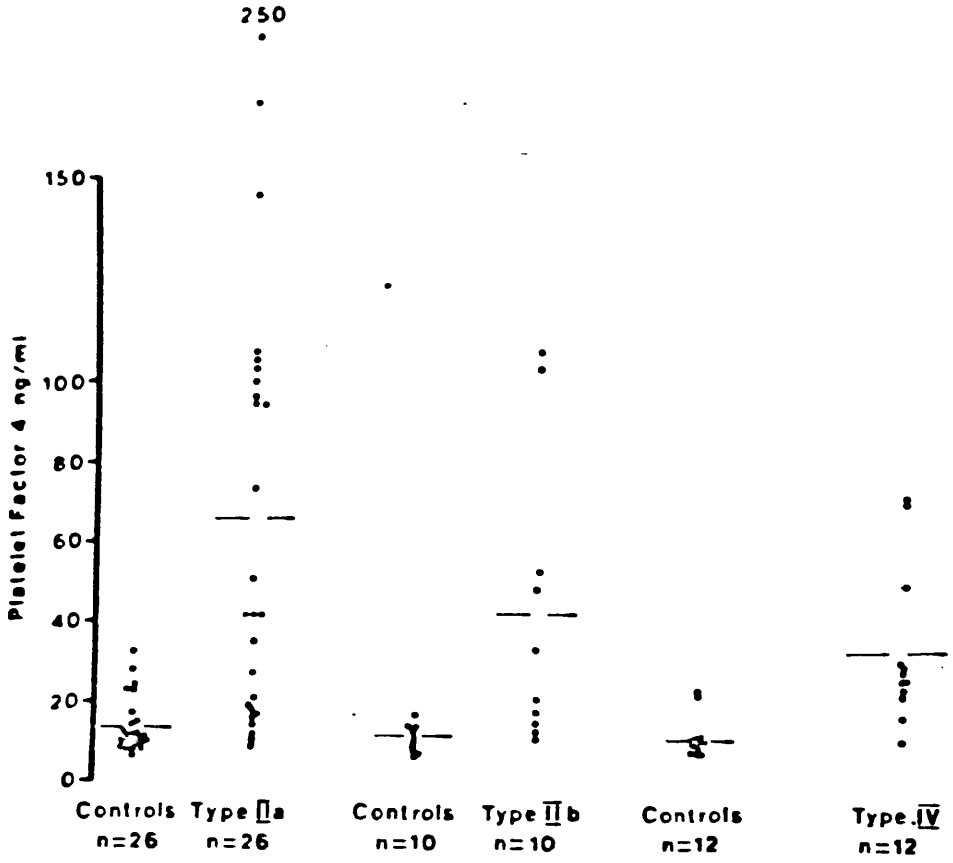

Figure 17

Mean (horizontal lines) and range of plasma platelet factor 4 (ngl/ml) in 48 patients with three types of hyperlipidaemia and age- and sex-matched controls. The difference (Student unpaired t-test) between the mean logarithmic values of patients and controls was highly significant ($P<0.001$ in patients with type IIa and type IV hyperlipidaemia and $P<0.002$ in those with type IIb hyperlipidaemia, respectively).

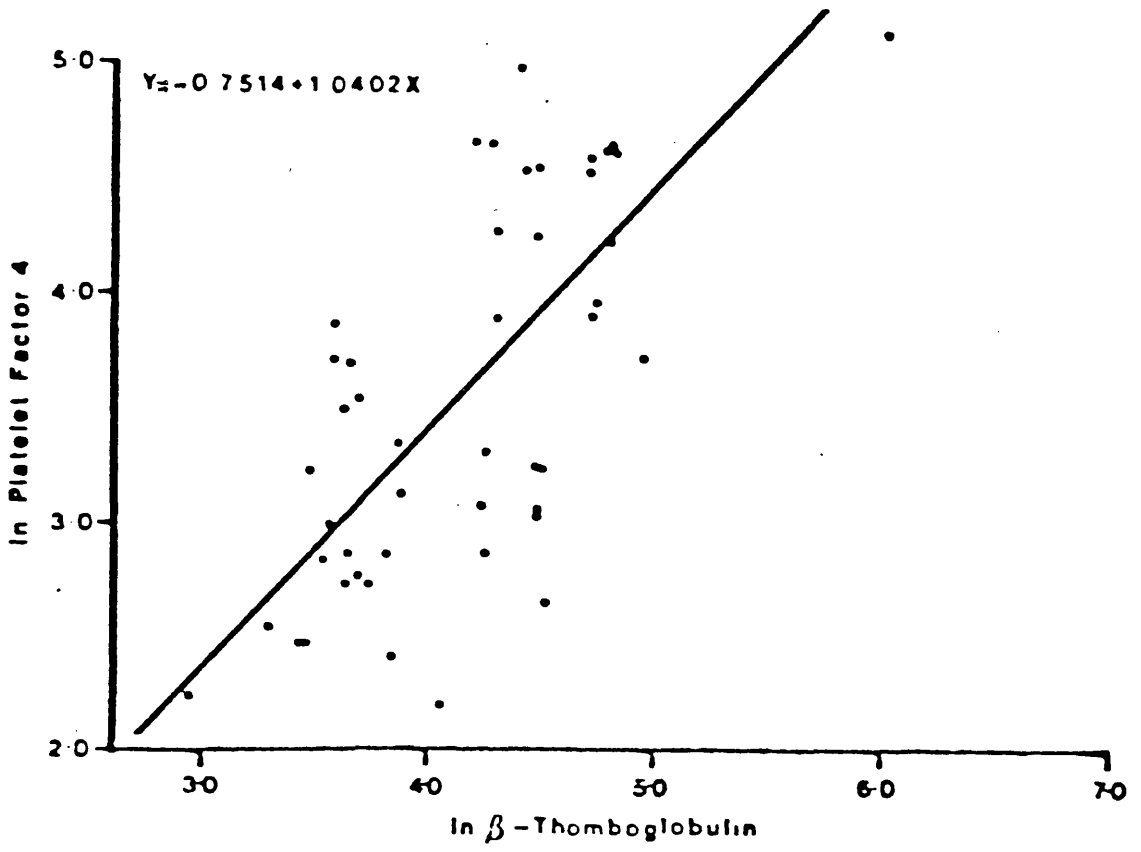

Figure 18

Linear regression analysis correlating plasma β-thromboglobulin values to platelet factor 4 in 48 hyperlipidaemic patients. ($r=0.6986$, $P<0.001$ and 95 percent confidence limits: $0.516 < r < 0.768$).

Statistically the difference between the mean logarithmic level of β-thromboglobulin of patients and controls was highly significant (Table10). The mean logarithmic β-thromboglobulin level was higher in patients with type IIb and type IV hyperlipidaemia than in those with type IIa hyperlipidaemia but this difference was not significant (Students unpaired t-test).

The mean plasma β-thromboglobulin level was also higher in the hyperlipidaemic patients than in the patients with peripheral vascular disease (mean 62.5 ng/ml, range 16-160 ng/ml but with much overlap. Statistically the difference between the mean logarithmic β-thromboglobulin value in these patients was highly significant ($P < 0.009$ for type IIa hyperlipidaemia, $P < 0.017$ for type IIb hyperlipidaemia and $P < 0.001$ for type IV hyperlipidaemia (Students unpaired t-test). However the mean logarithmic β-thromboglobulin level was significantly elevated in the 57 patients with peripheral vascular disease compared to that in age- and sex-matched controls ($P < 0.001$).

The mean plasma platelet factor 4 level was higher in 48 hyperlipidaemic patients studied compared to healthy controls (Figure 17, Table 10). Again considerable overlap was observed but 31 patients (16 out of 22 with type IIa hyperlipidaemia, 7 out of 10 with type IIb hyperlipidaemia and 8 out of 12 with type IV hyperlipidaemia), or 65%, the plasma platelet factor 4 level was beyond the upper range for controls. Statistically the difference between the mean logarithmic platelet factor 4 value of the patients and controls was highly significant (Table 10). There was no significant difference among the mean logarithmic platelet factor 4 values for the three groups of hyperlipidaemic patients.

Using linear regression analysis a strong positive correlation between β-thromboglobulin and platelet factor 4 was found in the hyperlipidaemic subjects ($P < 0.001$)(Figure 18). Similar correlations were obtained when regression analyses are performed separately in each type of hyperlipidaemia.

	Type IIa		Type IIb		Type IV	
	Controls	Patients	Controls	Patients	Controls	Patients
βTG*	28.8	91.2	27.1	106.8	27.9	119.7
Mean Range	8-65	19-390	10-66	27-300	9-54	30-324
ln βTG ± 1 SEM	3.24 ± 0.09	4.32 ±0.11	3.19 ± 0.12	4.42 ± 0.22	3.32 ± 0.10	4.53 ± 0.15
P		<0.001		<0.001		<0.001
PF4†	13.6	64.5	11.6	41.7	12.1	32.8
Mean Range	6.6-27.6	9-166	8-17	12-105	8-23	11-70
ln PF4 ± SEM	2.49 ± 0.08	3.74 ± 0.19	2.41 ± 0.1	3.42 ± 0.26	2.42 ± 0.10	3.35 ± 0.15
P		<0.001		<0.002		<0.001

NOTE: ln = natural logarithm (base e). P = student unpaired t-test comparing ln βTG or PF4 of patients to controls.
SEM = standard error of the mean.
* 69 patients
† 48 patients

Table 10

Plasma β-thromboglobulin (βTG) in ng/ml and platelet factor 4 (PF4) in ng/ml in hyperlipidaemia patients and age- and sex-matched controls.

Malondialdehyde Formation

The mean platelet malondialdehyde formation was higher in the patients than in the healthy controls (Figure 19 , Table 11). The overlap between patients with type IIa hyperlipidaemia and controls was almost total; however, in 5 of 9 patients with type IIb hyperlipidaemia and 7 of 16 patients with type IV hyperlipidaemia, malondialdehyde formation was beyond the upper range for controls (Figure 19). Statistically the difference between the mean logarithmic malondialdehyde value of the three groups of patients and the controls was significant Table 11). In addition there was a significant difference ($P < 0.016$) between the mean logarithmic malondialdehyde value of those with type IIb and type IV hyperlipidaemia compared to those with type IIa hyperlipidaemia.

There was a strong positive correlation between 16 logarithm values of plasma β-thromboglobulin and platelet malondialdehyde formation in type IV hyperlipidaemia ($r = 0.7660$, $P < 0.003$).

Clinically 25 out of the 69 hyperlipidaemic patients suffered from large vessel disease (ischaemic heart disease, peripheral vascular disease or chronic cerebrovascular disease). In these patients malondialdehyde formation together with β-thromboglobulin and platelet factor 4 values were slightly higher than in the remaining patients with no apparent vascular disease. However the difference between the two groups was not significant.

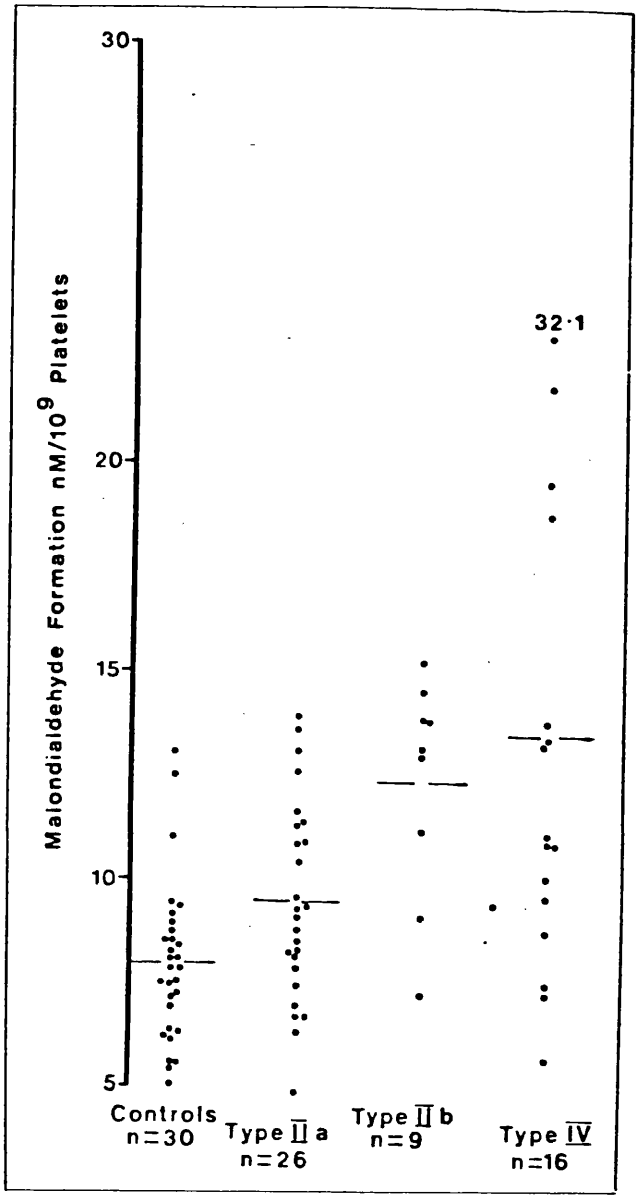

Figure 19

Mean (horizontal lines) and range of malondialdehyde formation (nmol/10^9 platelets) by washed platelets of 51 patients with three types of hyperlipidaemia and 30 controls. The difference (Student unpaired t-test) between the mean values of patients and controls was highly significant, ($P<0.001$ in patients with type IIb and type IV hyperlipidaemia, and $P<0.014$ in patients with type IIa hyperlipidaemia, respectively).

Subjects studied	No.	MDA		ln MDA±1 SEM	P*
		Mean	Range		
Controls	30	7.9	5.1-13	2.04 ± 0.04	...
Patients with hyperlipidaemia					
Type IIa	26	9.4	4.8-13.9	2.21 ± 0.05	<0.014
Type IIb	9	12.3	7.1-16.2	2.48 ± 0.09	<0.001
Type IV	16	13.4	5.6-32.1	2.49 ± 0.11	<0.001

NOTE: ln = natural logarithm (base e).
* Student unpaired t-test comparing patients to controls.

Table 11 Platelet malondialdehyde (MDA) formation in nmol/10^9 platelets in 51 hyperlipidaemic patients and 30 controls.

DISCUSSION

Studies in Diabetic Patients

Platelet-Specific Proteins

In recent years it has become possible to measure the platelet specific proteins β-thromboglobulin and platelet factor 4 (Moore *et al*, 1975; Niewiarowski *et al* 1969). These proteins are stored within the platelet alpha-granules (Niewiarowski, 1977) and are released to the surrounding plasma during platelet aggregation induced by thrombin collagen, ADP, adrenaline and various lectins (Holmsen *et al* 1969; Holmsen, 1975). β-Thromboglobulin is the most abundant platelet-specific protein with a platelet concentration $30\text{-}10^4$ times higher than in other tissues (Ludlam, 1979). Since β-thromboglobulin is cleared from human plasma with a half-life of 100 min at 37°C (Dawes *et al* 1978) an elevated level in platelet-poor plasma may be a useful indicator of enhanced *in vivo* platelet activation and 'release reaction'. In fact plasma β-thromboglobulin levels were shown to correlate closely to platelet lifespan (Ludlam, 1979). Platelet factor 4 on the other hand because of its very short half-life (Musial *et al* 1980) is less sensitive than β-thromboglobulin in detecting platelet secretion *in vivo* but it is a useful marker of artifactual secretion *in vitro* (Kaplan and Owen, 1981)

In the results reported in this thesis β-thromboglobulin levels in platelet-poor plasma were found to be highly significantly elevated in diabetic subjects compared to age and sex matched non-diabetic control subjects. Similar findings were observed for platelet factor 4 levels and the close correlation observed between β-thromboglobulin and platelet factor 4 levels both in diabetic subjects and control subjects suggested that the elevated β-thromboglobulin levels were not due to *in vitro* release reaction. Indeed great care was taken in these studies to follow

as carefully as possible the measures described by Zahavi *et al* (1980) to achieve accurate determination of β-thromboglobulin and platelet factor 4. Clean venepuncture was performed without venous stasis and the blood was collected into cooled anticoagulant containing tubes, separated immediately by centrifugation (2000 x g) at 4°C for 30 min to obtain platelet-poor plasma which was immediately frozen at -20°C to wait analysis.

Previous studies of β-thromboglobulin levels in diabetic subjects have yielded conflicting results. Whereas in one study no significant increase in β-thromboglobulin levels was found (Campbell *et al*, 1977) other studies revealed higher levels of β-thromboglobulin in diabetic patients (Burrows *et al*, 1978; Preston *et al*, 1978). Campbell *et al* (1977) had suggested that the reason for the discrepant findings of these studies was that in their study prostaglandin E_1 was added to the anticoagulant mixture whereas in the other studies this was not present in the anti-coagulant mixture. However Borsey *et al* (1980) measured β-thromboglobulin using four different techniques and although levels of β-thromboglobulin were lower when prostaglandin E_1 was present in the anticoagulant they remained elevated in diabetic subjects. In addition it is unlikely that this factor would be independent of the type of subject studied and would occur both in the diabetics studied as well as the healthy controls.

In the early studies of β-thromboglobulin levels in diabetic subjects the number of patients studied was small and they were not compared to age- and sex-matched control subjects. This is of importance as in some studies of β-thromboglobulin in non-diabetic populations a significant increase in levels has been seen with increasing age (Ludlam, 1979; Zahavi *et al*, 1980), although Dewar *et al* (1979) did not demonstrate this relationship. Also in one study there was a sex difference in old healthy

subjects (Zahavi *et al*, 1980). In this thesis the study of the platelet-specific proteins β-thromboglobulin and platelet factor 4 has been extended to a large group of diabetic patients compared to a group of non-diabetic healthy individuals well-matched for age and sex. The findings of elevated levels of β-thromboglobulin and platelet factor 4 in diabetic subjects provide further evidence that *in vivo* platelet activation and release reaction is enhanced in diabetes.

β-Thromboglobulin levels have been shown to be elevated in various types of vascular disease. Ludlam *et al* (1975) found elevated levels in patients presenting with subacute venous thrombosis. Stewart *et al* (1983) described significantly higher levels of platelet-specific proteins in patients with transient cerebral ischaemia and Handin *et al* (1979) found elevated levels in patients following myocardial infarction. Cella *et al* (1979) found elevated β-thromboglobulin levels in patients with peripheral vascular disease but not in patients with cerebrovascular disease. Because of these findings the diabetic subjects studied in this thesis were carefully examined to discover which of them had evidence of vascular complications. This was done to try and separate within the diabetic subjects studied effects on β-thromboglobulin due to pre-existing vascular disease and effects due to the altered metabolic state. Unfortunately present clinical methods cannot rule out early vascular disease, particularly large vessel disease, and until non-invasive imaging techniques are available, possibly involving nuclear magnetic resonance, complete exclusion of vascular disease will not be possible. The results for the platelet-specific proteins were analysed therefore within the diabetic subjects depending on the presence or absence of

complications. An interesting and important finding to emerge following this analysis was that those diabetics without clinical evidence of vascular disease still had highly significantly elevated β-thromboglobulin levels when compared to age and sex-matched controls. Those diabetics with clinical evidence of retinopathy had small but statistically significant elevations of β-thromboglobulin compared to diabetics without retinopathy. No difference was seen for β-thromboglobulin levels for diabetics with and without clinical large vessel disease. These findings suggest that abnormalities in *in vivo* platelet activation and release reaction may precede the development of vascular complications in the diabetic and imply that the abnormalities are related in some way to the abnormal metabolic state. This is in keeping with the findings of Preston *et al* (1978) who found elevated levels of β-thromboglobulin in a small group of newly diagnosed diabetic subjects. However there are other factors apart from hyperglycaemia in the altered metabolic state of diabetes which might directly affect platelet function or indirectly affect platelets by actions on vascular endothelium. In the present study glycosylated haemoglobin which gives an overall assessment of glycaemic control over a 4-6 week period (Bunn *et al* 1976) was measured as an assessment of glucose homeostasis in 62 of the diabetics studied. In these diabetics no correlation was found between glycosylated haemoglobin and levels of platelet specific proteins. In an early study of β-thromboglobulin in a mixed group of 72 diabetic subjects no correlation was found with simultaneous measurements of plasma glucose concentrations (Burrows *et al* 1978). Platelet factor 4 showed a significant positive correlation with plasma glucose concentration in a small group of insulin-dependent diabetics but no correlation was seen for β-thromboglobulin and no relationship was found for either of the platelet specific proteins in a larger group of non-insulin dependent diabetics (Davi *et al* 1982).

The findings in this thesis of no correlation between platelet-specific protein levels and glycosylated haemoglobin concentrations are in agreement with findings from other studies (Davi *et al*, 1982; Mathews *et al*, 1979).

Equally conflicting results with regard to glycaemic control and platelet-specific protein levels have been reported in longitudinal studies. In 9 newly diagnosed non-insulin-dependent diabetics mean β-thromboglobulin levels fell from 74 ng/ml (range 24-277 ng/ml) to 39 ng/ml as treatment with diet and hypoglycaemic agents reduced mean blood glucose concentrations from 14.3 to 8.5 mmol/l (Preston *et al*, 1978). However in longitudinal studies of moderately well controlled insulin-dependent diabetics treated with constant subcutaneous insulin for 2 weeks (Delamothe and Betteridge, 1985) or 4-8 weeks (Rosove *et al*, 1984) which led to significant lowering of mean plasma glucose and glycosylated haemoglobin levels, no changes were observed in platelet factor 4 or β-thromboglobulin levels. On the other hand in a short term study using the artificial pancreas, normalisation of blood glucose for 48 h led to a significant fall in β-thromboglobulin (Voisin *et al*, 1983).

As has been seen in the introduction to this thesis hyperlipoproteinaemia is common in diabetes mellitus. Therefore in this thesis correlations were sought between platelet-specific protein levels and fasting total serum cholesterol and serum triglyceride and the individual lipoproteins. These measurements were available in 49 of the diabetic subjects studied. Small but statistically significant correlations were found between β-thromboglobulin levels and total triglyceride, VLDL-triglyceride, LDL-cholesterol and HDL/total cholesterol ratio. The correlations were positive apart from the correlation with HDL/total cholesterol which was negative. These findings suggest that changes seen in platelet

specific proteins in diabetes might be secondary to associated hyperlipoproteinaemia. In addition these correlations between individual lipid and lipoprotein concentrations and platelet-specific proteins may explain the changes of platelet specific proteins with instigation of diabetic therapy (Preston *et al* 1978), as lipid levels would be expected to be elevated in uncontrolled diabetes and to fall with improved control (Simpson *et al* 1979; Paisey *et al* 1978). As will be discussed later in this thesis the lipid environment of the platelet may directly affect platelet function. In addition recent experimental work has suggested that lipoproteins (VLDL) obtained from diabetic animals (streptozotocin diabetic rats) may be more toxic to cultured porcine endothelial cells than lipoproteins obtained from control animals. This effect which may be due to increased lipid peroxide levels in diabetes was reversed by insulin treatment (Arbogast *et al* 1982). Other studies have shown increased lipid peroxide levels in lipoproteins isolated from diabetic patients (Nishigaki *et al* 1981) and animals (Higuchi, 1982). These findings suggest that lipoprotein abnormalities in the diabetic state may also indirectly affect platelet function by damaging vascular endothelium. There is as yet no direct measure of endothelial damage in the intact animal. However, some recent studies have measured plasma levels of Factor VIII-related antigen as an indirect measure of endothelial cell damage together with β-thromboglobulin levels. Rak *et al* (1983) found that plasma levels of both proteins were elevated in diabetics including children. It is difficult to determine therefore whether endothelial damage preceded the evidence of enhanced platelet release reaction or not. Janka *et al* (1983) studied Factor VIII-related antigen and β-thromboglobulin levels in groups of diabetics with and without vascular disease. These authors found an increase in Factor VIII-related antigen in all groups of diabetics whereas β-thromboglobulin

was only elevated in diabetics with retinopathy. This study suggests that endothelial damage (as assessed by Factor VIII-related antigen levels) may precede evidence of *in vivo* platelet aggregation but obviously further work needs to be done in this area.

So far this discussion has dealt with the elevated levels of platelet specific proteins purely as a likely measure of enhanced *in vivo* platelet release reaction in diabetic subjects. However, do these platelet specific proteins have a physiological role in their own right? The important platelet mitogen, platelet-derived growth factor which is an alpha granule constituent along with β-thromboglobulin and platelet factor 4 has been discussed in the introduction to this thesis. Both β-thromboglobulin and platelet factor 4 have anti-heparin activity. However it is doubtful whether this property of the platelet specific proteins has any physiological importance since the concentration of heparin is probably insignificant (Niewiarowski and Paul, 1981). Nevertheless these proteins also react with other glycosaminoglycans such as heparin sulphate (Barber *et al* 1972; Niewiarowski *et al* 1979). Heparin sulphate occurs on the surface of many cells including endothelial cells (Lindahl and Hook, 1978) and there is evidence that both platelet factor 4 (Busch *et al* 1980) and β-thromboglobulin (Hope *et al* 1979) bind to cultured endothelial cells. In addition β-thromboglobulin appears to inhibit prostacyclin synthesis in bovine aortic endothelial cells (Hope *et al* 1979). Platelet factor 4 appears to act as an inhibitor of collagenase which suggests a possible physiological role in connective tissue metabolism (Hiti-Harper *et al* 1978). However the possible physiological roles for β-thromboglobulin and platelet factor 4 remain to be determined.

Malondialdehyde Formation

As discussed in the introduction to this thesis prostaglandins, thromboxanes and their precursor arachidonic acid are critical substances in platelet metabolism (Samuelson, 1977). Earlier studies of the prostaglandin pathway in platelets from diabetic subjects measured the classical prostaglandins. Thus Halushka *et al* (1977) demonstrated that diabetic platelets synthesized greater amounts of prostaglandin E_2 in response to various agonists compared to platelets from matched control subjects. Most of the patients studied were free of vascular disease. Subsequently increased serum concentrations of the prostaglandins E_2 and $F_{2\alpha}$ were demonstrated in the serum of diabetic children (Chase *et al*, 1979). Although these findings indicate increased activity of the platelet prostaglandin pathway in diabetic subjects they do not explain the increased platelet aggregation as these prostaglandins are not in themselves aggregatory.

The formation of the potent aggregating substance thromboxane A_2 in diabetic platelets has to be assessed indirectly because it is very labile with a half-life of approximately 30 sec in aqueous solution (Hamberg *et al*, 1975). In this thesis malondialdehyde formation was used as an indirect measure of thromboxane A_2 production in platelets stimulated by arachidonic acid. Malodialdehyde is a stable product of the conversion of endoperoxides to thromboxane A_2 by the microsomal enzyme thromboxane synthetase and is formed in equimolar amounts (Diczfalusy *et al*, 1977). However malondialdehyde may also be formed non-enzymatically from prostaglandin endoperoxides. Stuart *et al* (1979) had previously shown that platelets obtained from pregnant diabetic women synthesized increased amounts of malondialdehyde and McGuire *et al* (1978) had demonstrated increased malondialdehyde production *in vitro* by platelets from patients with diabetes mellitus. In the present study a highly significant increase was observed in

malondialdehyde formation in platelets from diabetic subjects compared to controls. This finding suggests that diabetic platelet may have enhanced activity of the thromboxane synthetase pathway when stimulated with arachidonic acid. Janka *et al* (1983) measured malondialdehyde formation in platelets stimulated by n-ethyl-maleimide (1 mM) from diabetics with and without clinical evidence of retinopathy. These authors found that platelets from retinopathy patients formed more malondialdehyde than platelets from patients without retinopathy but there was no difference between non-retinopathy patients and controls. In the present study there were insufficient patients with retinopathy in whom malondialdehyde formation was measured to make a meaningful analysis. It is possible that the differing results between the study of Janka *et al* (1983) and the present study relate to the different techniques used.

There are several studies in the literature which have assessed thromboxane A_2 formation by the measurement of thromboxane B_2 by radioimmunoassay. Thromboxane A_2 spontaneously rearranges to form thromboxane B_2 which is stable and considerably less potent (Friedman *et al* (1979). Using this technique Butkus *et al* (1980) measured thromboxane B_2 generation in platelets in response to exogenous arachidonic acid quantitated by electron-capture gas chromatography in a group of 180 insulin-dependent diabetics. Although platelet thromboxane generation was elevated in diabetics without clinical evidence of vascular disease the difference did not reach statistical significance. However those diabetics with retinopathy, neuropathy, nephropathy, peripheral vascular disease or coronary vascular disease did show significantly enhanced platelet thromboxane generation compared to controls (Butkus *et al*, 1980). Ziboh *et al* (1979) also found that those diabetics with complications

had higher platelet thromboxane generation than diabetics without complications. However in the study of Halushka *et al* (1981) thromboxane generation appeared to be increased in diabetics without complications. In addition evidence of increased thromboxane generation comes from studies using experimental animals. For instance the spontaneously diabetic BB-Wistar rat (Subbiah *et al* 1980 and the streptozotocin diabetic rat (Johnson *et al* 1980). In a recent study Butkus *et al* (1982) have assessed platelet thromboxane production in insulin-dependent diabetics with and without coronary artery disease. Platelets from diabetics with coronary artery disease had higher thromboxane production than those without coronary disease and diabetic platelets in general produced more thromboxane than non-diabetic platelets. It would appear therefore from this and the other studies cited that the weight of evidence supports the finding reported in this thesis of increased thromboxane production in the platelets of some diabetic subjects whether measured as malondialdehyde formation or thromboxane B_2 levels. In addition those diabetics with vascular complications tend to show higher levels of thromboxane production than those diabetics free of complications.

Some support for the concept that increased thromboxane synthesis in platelets from diabetic subjects results from the altered metabolism of the diabetic state rather than being secondary to pre-existing vascular disease comes from studies which have correlated thromboxane production with diabetic control. Halushka *et al* (1981) found a significant positive correlation between fasting plasma glucose concentrations and platelet thromboxane production stimulated by arachidonic acid in a group of 15 diabetics. In addition platelet thromboxane synthesis was lower in a group of diabetics treated with constant subcutaneous insulin infusion for 6 months compared to a control group on conventional therapy (McDonald,

et al 1982). However when normal platelets were incubated with increasing glucose concentrations *in vitro*, thromboxane production was unaffected (Best *et al* 1979) and the findings of Halushka *et al* (1981) and McDonald *et al* (1982) have not been supported by a recent study of platelet thromboxane generation before and after constant subcutaneous insulin infusion (Jackson *et al* 1984). These authors measured platelet thromboxane generation in response to arachidonic acid and collagen in a group of 11 diabetic patients with neuropathy. Prior to the start of therapy with constant subcutaneous infusion, thromboxane production was enhanced in the diabetics compared to controls and this enhanced production persisted after intensive therapy for 16 weeks. On the other hand collagen stimulated thromboxane production was similar in diabetics and controls prior to insulin infusion apart from at the lowest collagen concentration (0.5 µg/ml) when thromboxane production was in fact lower than that seen in control subjects. After insulin infusion this apparent subnormal thromboxane production returned to normal (Jackson *et al* 1984). This finding may be due to a reduced level of arachidonic acid in platelet membrane phospholipids of diabetic subjects as described by Jones *et al* (1983). This reduced level of arachidonic acid showed a negative correlation with glycosylated haemoglobin (Jones *et al* 1983). The finding therefore of an increase in collagen stimulated thromboxane production by insulin infusion could be explained by an increased availability of endogenous arachidonic acid from membrane phospholipids (Jackson *et al* 1984).

Platelet Sensitivity to Prostacyclin

An important and interesting finding to emerge from the studies undertaken for this thesis was that platelets from some diabetic patients appeared to have a marked reduction in sensitivity to the anti-aggregatory prostanoid, prostacyclin. This sensitivity was calculated as the amount of prostacyclin necessary to produce a 50% inhibition of ADP-induced-aggregation, the prostacyclin ID_{50}. Some 40% of the diabetics studied had prostacyclin ID_{50} levels greater than the highest value obtained in control subjects. The control subjects used in this study were younger than the diabetic population and it is possible that the observed differences in sensitivity to prostacyclin between the two groups merely reflects age difference. However no correlation was found between the sensitivity of the platelets to prostacyclin with age in either diabetics or controls.

When the diabetic group was analysed according to the presence or absence of clinical evidence of large vessel disease, those diabetics with vascular disease had highly significantly decreased platelet sensitivity to prostacyclin. The sensitivity to prostacyclin of platelets from diabetics without clinical evidence of vascular disease compared to controls was diminished but this did not reach statistical significance. Therefore it is possible that the observed differences in prostacyclin sensitivity may reflect the presence of vascular disease in the diabetic group. However the findings reported in this thesis from studies using the electronic platelet aggregometer where diabetic subjects were deliberately chosen because they were free of vascular disease support the finding of decreased sensitivity of diabetic platelets to prostacyclin. The findings of decreased sensitivity of diabetic platelets to prostacyclin in this thesis have been confirmed by a study from Japan (Onodera *et al* 1982).

The reduced sensitivity of some diabetic platelets to prostacyclin remains to be explained. A possible explanation would be that the diabetic environment might affect the platelet receptors for prostacyclin. However prostacyclin receptors have been studied in diabetic platelets and no change in the number or affinity of membrane receptors was found (Dollery *et al* 1983; Shepherd *et al* 1983). In addition, prostcyclin-dependent activation of adenylate cyclase was similar in diabetics and controls (Dollery *et al* 1983; Janka *et al* 1983). However it has to be remembered that only 40% of diabetics showed reduced sensitivity to prostacyclin and in the above studies receptor binding and affinity and adenylate cyclase activity were not studied in platelets shown to have decreased prostacyclin senstivity.

Whole Blood Platelet Aggregation

Using the Chronolog Model 540 electronic aggregometer platelet responses to arachidonic acid, collagen and prostacyclin were studied in whole blood samples from diabetic subjects. Diabetic subjects showed enhanced platelet aggregation to both collagen (1 μg/ml) and arachidonic acid (1 mmol/l) and diminished sensitivity to prostacyclin.

This method of measuring platelet aggregation which was recently developed by Cardinal and Flower (1980) may have advantages over the turbidometric method of Born (1962). When two electrodes are suspended in stirred whole blood, a monolayer of platelets forms and the conductance between the electrodes is constant. When an aggregation agent is added, however, platelets aggregate to the monolayer and an accretion of platelets occurs between the two electrodes. This resulting increase in impedence between the electrodes can be observed on a pen recorder. The important difference between this technique and that of optical platelet aggregation is that platelet behaviour can be studied immediately after sampling because

the centrifugation step (to produce PRP) is avoided. In addition platelet responses are studied in the presence of formed blood elements and other potentially important but highly labile factors all of which may influence platelet behaviour. Before platelet aggregation can be studied using the optical method PRP has to be prepared. This may take 15-20 min and during this time labile modulators of platelet aggregation such as prostacyclin and thromboxane A_2 may have decayed to a great extent. In addition formed elements of blood may potentially affect platelet function by releasing platelet-active substances such as prostacyclin from leucocytes (Blackwell *et al* 1978) and ADP from red blood cells (Juhan *et al* 1982). The centrifugation step to prepare PRP may affect the population of the platelets studied as some platelets may be discarded with the red cells. For the above reasons the electronic whole blood aggregometer as described by Cardinal and Flower (1980) may be a more physiological approach to the study of platelet aggregation.

The results presented in this thesis using the new technique confirm previous studies of enhanced platelet aggregation in diabetic subjects using the optical aggregometer (discussed in the introduction). Care was taken in the choice of diabetic subjects for the study because as far as possible it was felt important to exclude diabetics with pre-existing vascular disease as the point of the study was to determine whether platelet abnormalities were related to the metabolic abnormalities in diabetics rather than pre-existing vascular disease. Many of the early studies of platelet aggregation failed to make this distinction (see introduction). Although the presence of vascular disease cannot be completely excluded by thorough clinical assessment these results do suggest that the platelet abnormalities in this diabetic group are due to the diabetic state. Further evidence to support this interpretation

comes from the studies with prostacyclin. Platelet aggregation in whole blood from diabetic subjects showed reduced sensitivity to the anti-aggregatory effects of prostacyclin and a significant direct correlation was present between glycaemic control as assessed by levels of glycosylated haemoglobin and the prostacyclin ID_{50}. The reduced sensitivity of diabetic platelets to prostacyclin is in agreement with other results presented in this thesis using PRP in the optical aggregometer.

It is tempting to speculate that hyperglycaemia may directly affect platelet function by glycosylation of the platelet membrane but this remains to be determined. Alternatively those diabetics with higher glycosylated haemoglobin levels may have other metabolic abnormalities such as hyperlipoproteinaemia as previously described in this thesis. Alternatively the altered platelet responsiveness in diabetes may be secondary to alterations to the vascular endothelium. Unfortunately it was not possible to make an indirect measurement of endothelial function in these patients by measurement of Factor VIII related antigen for instance.

The whole blood aggregometer has certain drawbacks compared to the optical aggregometer - it is certainly more difficult technically! In addition it is unable to record platelet shape change (Cardinal and Flower, 1980). The failure to show differences between diabetics and control subjects with the higher dose of collagen (5 μg/ml) may also be related to the fact that the impedance aggregometer is more sensitive to smaller platelet aggregates than to the larger aggregates which would have been induced by the high dose collagen (Ingerman-Wojenski *et al*, 1982).

Prostacyclin Production by Tissues from Pregnant Diabetic Rats

Discovery of the highly important cyclo-oxygenase product prostacyclin (Moncada *et al*, 1976) which is the most potent endogenous anti-platelet aggregatory agent known has stimulated considerable interest in the possible effects of the diabetic state on its metabolism. Harrison *et al* (1978) found decreased prostacyclin production in aortic rings taken from streptozotocin diabetic rats. This original observation has been confirmed and extended by other workers. Silberbauer *et al* (1980) induced a mild form of diabetes in Göttingen miniature pigs by repeated administration of streptozotocin. The amount of prostacyclin generated (assayed by inhibition of ADP-induced aggregation) by vascular tissue was significantly reduced in these mildly diabetic animals (Silberbauer *et al*, 1980). Recently Kern and Engerman (1984) have demonstrated a reduction in aortic 6-oxo-prostaglandin $F_{1\alpha}$ production in diabetic dogs. The present study confirms these earlier findings in that prostacyclin production assayed by inhibition of ADP-induced aggregation was significantly reduced in aortic ring preparations. In addition it appears from the present study that the reduction of prostacyclin synthesis is related to the duration of the diabetic state. No significant difference was seen in prostacyclin production by tissues from control and acutely diabetic animals (diabetic for up to 15 days). On the other hand, a highly significant reduction in prostacyclin synthesis was observed in chronically diabetic rats (diabetic for up to 8 weeks). Similar findings have been reported by other workers. Rogers and Larkins (1981) studied the time course of diabetes on the production of 6-oxo-prostaglandin $F_{1\alpha}$ (the stable metabolite of prostacyclin) in aortic rings from streptozotocin diabetic rats. After two weeks of diabetes, 6-oxo-prostaglandin $F_{1\alpha}$ production did not differ between control and diabetic rats. However, 4 and 7 weeks

after the induction of diabetes 6-oxo-prostaglandin $F_{1\alpha}$ production was significantly reduced even though the degree of hyperglycaemia and hypoinsulinaemia was the same as at 2 weeks. The reduction in 6-oxo-prostaglandin $F_{1\alpha}$ was not merely related to weight loss in the diabetic animals as in rats on a restricted dietary intake which led to weight loss, 6-oxo-prostaglandin $F_{1\alpha}$ production remained unaffected.

In the present study an interesting finding in the pregnant diabetic rats was that even in chronically diabetic rats there was no reduction in prostacyclin production by myometrial tissue. It therefore appears that the effect of the diabetic state on prostacyclin production shows a degree of selectivity. However in other studies a reduced output of prostacyclin has been described in renal cortical tissue from chronically diabetic rats (Harrison *et al*, 1978). This reduction may reflect decreased prostacyclin production by renal blood vessels as the renal cortex is a highly vascular tissue. In the myometrial tissue which showed no change in prostacyclin production secondary to the diabetic state the contribution of the blood vessels is minimal, the myometrial cells being the predominant source of prostacyclin (Williams *et al*, 1978).

The mechanism of the decreased prostacyclin production in vascular tissue from diabetic animals remains to be determined. It may be related to prostaglandin precursor availability. Mercurio *et al* (1966) found depression of the microsomal desaturation of linoleic to gamma-linoleic acid in the alloxan diabetic rat and Jones *et al* (1983) have found low phospholipid arachidonic acid levels in platelets from diabetics and an inverse correlation between the percentage of arachidonic acid and glycosylated haemoglobin concentrations. Other workers (Kalofoutis and Lekakis, 1981) found a higher percentage of arachidonic acid in diabetic platelets compared to controls. On the basis of altered substrate availability, however, it is difficult to envisage why the reduction in

prostacyclin synthesis is selective for vascular tissue. Another alternative explanation for the selectivity of prostacyclin reduction by the diabetic state could relate to differences in phospholipase activities in the different tissues which could alter precursor availability and hence prostacyclin synthesis. Currently there is no information on this. A likely explanation for the reduced vascular prostacyclin production in diabetes may well be the increased level of lipid peroxides which are known to be present in diabetic animals (Higuchi, 1982) and diabetic patients (Nishigaki *et al*, 1981). These peroxides are potent inhibitors of prostacyclin synthesis (Salmon *et al*, 1978). This is supported by two recent studies which have examined the effect of the diabetic environment on prostacyclin production by cultured human endothelial cells (Paton *et al*, 1982; Patel and McEvoy, 1983). When these cells were incubated with serum from diabetic patients prostacyclin production was inhibited. The nature of the inhibitory factor or factors in the diabetic serum is not understood but a likely contender would be lipid peroxides. This possibility is supported by the findings of Karpen *et al* (1982). These authors found that vitamin E treatment of diabetic rats reduced plasma lipid peroxide levels and along with this vascular prostacyclin production and thrombin-induced platelet thromboxane formation were restored to normal levels.

Some workers have been able to study the effect of diabetic treatment on prostacyclin production in experimental animals. Harrison *et al* (1980) demonstrated that the reduced prostacyclin release (measured by bioassay) from aorta and renal cortex of streptozotocin-induced diabetic rats was restored to that seen in control non-diabetic rats by chronic (eight days) treatment with insulin. However no effects were seen on prostacyclin production immediately after the start of insulin treatment. It is likely that chronic insulin treatment is necessary to allow endothelial cell loss

or damage to be repaired (Harrison *et al*, 1980). These findings have been extended in the streptozotocin diabetic rat by Rogers and Larkin (1981). These authors measured the production of 6-oxo-prostaglandin $F_{1\alpha}$ by rat aorta. They demonstrated that the reduced production of 6-oxo-prostaglandin $F_{1\alpha}$ seen in chronically diabetic rats (4-6 weeks) could be restored by a dose of insulin (8 units/kg/day) that only partially corrected plasma glucose levels and body weight.

It is obviously difficult to study arterial prostacyclin production in diabetic subjects. However in a small study of vascular tissue removed at operation prostacyclin generation was found to be reduced in tissue from diabetics (Johnson *et al*, 1979). Furthermore, Silberbauer *et al* (1979) showed that vein biopsies obtained from insulin-dependent diabetics generated significantly less prostacyclin compared to age- and sex-matched controls. On the other hand Davis *et al* (1981) found that venous prostacyclin production was lower in a small group of diabetics with nil or minimal retinopathy compared to diabetics with proliferative retinopathy. This finding albeit in very small numbers, would seem to suggest that reduced prostacyclin production may not have a role in the development of microvascular disease. However it is likely that this finding may indicate increased prostacyclin production in response to vascular injury (Dollery *et al*, 1983).

Attempts have been made to measure plasma prostacyclin levels indirectly by measurement of 6-keto-prostaglandin $F_{1\alpha}$ by radio immunoassay. Using this method conflicting findings have been reported in studies of diabetic subjects (Dollery *et al*, 1979; Davis *et al*, 1979; Davis *et al*, 1981) and it is now generally accepted that there may be considerable non-specific interference in assaying this metabolite in plasma which may explain the different findings (Dollery *et al*, 1983; Greaves and Preston, 1982).

Studies in Hyperlipidaemic Patients

Platelet Specific Proteins

The major findings from the study of hyperlipidaemic subjects was that the platelet specific proteins, β-thromboglobulin and platelet factor 4 were highly significantly elevated in the subjects with primary hyperlipidaemia compared to age and sex-matched controls. Although, as with the studies in the diabetic subjects, there was much overlap between the control and patient groups 65% of the patients had levels of platelet specific proteins higher than the highest level seen in the control subjects. Again, as in the diabetic studies, there was a strong correlation between β-thromboglobulin levels and platelet factor 4 levels suggesting that both proteins were released from the same platelet pool and at the same rate.

Hyperlipidaemic subjects as discussed in the introduction to this thesis are at risk from premature vascular disease. It is likely therefore that the hyperlipidaemic subjects studied for this thesis as a group had more vascular disease (sub-clinical) than the age and sex-matched controls. For this reason and the fact that β-thromboglobulin is known to be elevated in a variety of vascular conditions (Ludlam *et al* 1975; Stewart *et al* 1983; Handin *et al* 1978; Cella *et al* 1979) an extra control group of 57 patients with known peripheral vascular disease was included in this study. β-Thromboglobulin levels in this group of vascular disease patients were elevated as expected when compared to the healthy control group. However in all types of hyperlipidaemic patients studied β-thromboglobulin levels were significantly higher than in the vascular disease patients. This important finding strongly suggests that the evidence of enhanced *in vivo* release reaction as evidenced by the elevated β-thromboglobulin levels is related in some way to the hyperlipidaemic state.

The results reported here support previous studies of abnormal platelet function in hyperlipidaemic subjects (Colman, 1978; Shattil *et al* 1977; Carvalho *et al* 1974; Joist *et al* 1979; Tremoli *et al* 1979; Nordoy and Rodset, 1971) which have measured *in vitro* platelet aggregation, platelet factor 4, plasma antiheparin activity and a bleeding time test. Carvalho *et al* (1974) performed *in vitro* platelet aggregation studies on patients with familial type II hyperlipoproteinaemia (familial hypercholesterolaemia). Platelets from these subjects were found to be more sensitive to the aggregatory agonists adrenaline, collagen and ADP when compared to platelets from control subjects. In addition the platelet release reaction measured *in vitro* by the release of total nucleotides in response to the same three aggregating agents was increased. Eight of the patients studied had clinical evidence of atherosclerosis and the control subjects used were somewhat younger which may have to a certain extent influenced the results. However it does appear from the present study that these early *in vitro* findings of Carvalho *et al* (1974) are confirmed. On the other hand an interesting difference does emerge between the present study and that of Carvalho *et al* with regard to subjects with type IV hyperlipoproteinaemia (elevated VLDL levels). Platelets from 11 males with the type IV abnormality showed no evidence of increased sensitivity to aggregating agents *in vitro* in the early study. However in the present study reported in this thesis highly significant elevations of platelet specific proteins were seen in the patients with type IV hyperlipoproteinaemia. In this respect it is interesting that Joist *et al* (1979) who used the template bleeding time (as described by Mielke *et al* 1969) as a measure of platelet function *in vivo* found shorter template bleeding times in 16 patients with type IV hyperlipoproteinaemia. In addition increased *in vitro* aggregation has been described in a more recent study but only to adrenaline (Shastri *et al* 1980). However Hassall

et al 1983 who examined the relationship of *in vitro* aggregation to adrenaline, ADP, collagen and thrombin to prevailing lipid and lipoprotein concentrations in a normal male population found little relationship between VLDL and platelet sensitivity except for a reduced adrenaline response in subjects in the lowest quintile for VLDL concentrations. In contrast total and LDL cholesterol concentrations did appear to influence platelet sensitivity to adrenaline and to a lesser extent ADP (Hassall *et al*, 1983). It would appear therefore that overall increased *in vitro* platelet aggregation appears to be more strongly related to LDL than VLDL. However Aviran and Brook (1983) have provided evidence for a direct effect on *in vitro* platelet function for both LDL and VLDL. These workers incubated the purified lipoproteins with gel-filtered platelets for 30 min at 37°C and demonstrated that VLDL and LDL increased thrombin-induced platelet aggregation and [^{14}C] serotonin release induced by adrenaline, ADP and thrombin. High VLDL levels leading to the laboratory classification of type IV hyperlipoproteinaemia may be caused by several pathological processes and VLDL composition as well as concentration may be affected. It is likely therefore that the variable results in the literature relating VLDL levels to platelet function may be related to these differences in VLDL.

Further evidence for the relationship of LDL to platelet function has come from a prospective study of a small group of patients with familial hypercholesterolaemia treated by plasmaphoresis (Brook *et al*, 1983). These authors demonstrated that following reduction of cholesterol levels after plasmaphoresis there was a concurrent reduction in platelet aggregation and [^{14}C] serotonin release.

The mechanism of cholesterol-induced effects on platelet function has received considerable attention. Shattil *et al* (1975) studied the effect of the plasma lipid environment of platelets on platelet sensitivity to

aggregating agents *in vitro*. Shattil *et al* (1975) incubated normal platelets with "cholesterol-rich" liposomes which led to a substantial increase in platelet cholesterol concentration (39.2% increase after 5 h). The bulk of this increase in cholesterol (55%) was associated with the membrane fraction. Along with these changes in cholesterol concentration the cholesterol-rich platelets showed enhanced sensitivity to adrenaline and ADP measured by aggregometry and [^{14}C] serotonin release. Conversely platelets incubated with "cholesterol-poor" liposomes showed a reduced cholesterol content (24% reduction) and a significantly reduced sensitivity to ADP. On the basis of these findings Shattil *et al* (1975) postulated that cholesterol enrichment of the platelet membrane might affect the membrane fluidity and consequently the sensitivity to aggregating agonists. In support of these findings the same workers (Shattil *et al*, 1977) found that platelets from patients with type II hyperlipoproteinaemia had a higher cholesterol/phospholipid ratio and other workers have found an elevated platelet-free cholesterol in patients with the same condition (Miettinen, 1974). In addition Shattil and Cooper (1976) described increased platelet membrane microviscosity following incubation of platelets with "cholesterol-rich" liposomes and Sinha *et al* (1977) found an impaired response of the membrane associated enzyme adenylate cyclase to the stimulating effect of various agents including prostaglandin E_1 as shown by a decrease in production of cyclic AMP. More recent studies have demonstrated that increases in the cholesterol/phospholipid ratio of platelets resulted in increased thrombin-induced aggregation and increased liberation of arachidonate (Worner and Patscheke, 1980). Jakubowski *et al* (1980) failed to show an increase in the cholesterol/phospholipid ratio in platelets obtained from 17 hypercholesterolaemic subjects compared to 17 age- and sex-matched controls. These hypercholesterolaemic subjects had lower

plasma cholesterol levels than the subjects studied by Shattil *et al* (1977). However Jakubowski *et al* (1980) did show that the platelets from the hypercholesterolaemic subjects had an impaired ability to accumulate cyclic AMP compared with controls when challenged with prostacyclin or prostaglandins D_2 and E_1. The failure of these authors to demonstrate quantitative changes in the cholesterol/phospholipid ratio of whole platelets does not rule out changes in membrane lipids as this may be masked if only the total platelet lipids are studied (Shattil *et al*, 1977). These findings of Jakubowski *et al* (1980) of an impaired ability of whole platelets to accumulate cyclic AMP in response to prostacyclin do not appear to be explainable by changes in either affinity or number of prostacyclin receptors coupled to adenylate cyclase as Colli *et al* (1983) could not demonstrate any differences between basal and prostacyclin stimulated adenylate cyclase activity in washed platelet membranes from subjects with hypercholesterolaemia compared to controls. It is interesting therefore that the same authors showed decreased sensitivity of the hypercholesterolaemic platelets to the antiaggregatory effects of prostacyclin. The explanation for these findings remains to be clarified (Colli *et al*, 1983).

From the findings reported in this thesis of evidence of enhanced *in vivo* platelet release reaction in patients with hyperlipoproteinaemia together with the results of other studies outlined above it would appear that the lipid environment may have a direct effect on the metabolic responses of the platelet and these lipid/platelet interactions may well be of importance in the pathogenesis of atherosclerosis.

Malondialdehyde Formation

Further evidence for the importance of lipid/platelet interactions comes from results presented in this thesis of increased malondialdehyde formation by washed platelets from hyperlipidaemic subjects. Malondialdehyde is a stable end-product of the prostaglandin pathway as discussed previously and was used as a convenient marker for the assessment of prostaglandin synthesis and particularly thromboxane synthesis. Increased malodialdehyde formation was found in both subjects with hypercholesterolaemia and hypertriglyceridaemia. These findings are in agreement with a small study reported by Tremoli *et al* (1979). These workers studied washed platelets from 4 patients with hypercholesterolaemia in response to stimulation with arachidonic acid (20 μmol/l). Thromboxane B_2, the stable hydrolysis product of thromboxane A_2, measured by radioimmunoassay was significantly higher in hypercholesterolaemic platelets compared to controls and there was a highly significant positive correlation between platelet thromboxane B_2 production and plasma cholesterol levels.

Support for these findings and evidence of a direct relationship between cholesterol and thromboxane synthesis has come from *in vitro* studies. Beitz *et al* (1983) have studied the effect of LDL isolated from healthy male volunteers on malondialdehyde formation as a marker of the activity of thromboxane synthetase by frozen human platelets. They found there was a dose-dependent stimulation of malondialdehyde formation from prostaglandin H_2 by LDL (0.5 - 2.0 mg LDL-cholesterol/ml) whereas no effect was seen with albumin or HDL (Beitz *et al*, 1983). In addition Stuart *et al* (1980) altered platelet cholesterol content by incubation with "cholesterol-rich" or "cholesterol-poor" liposomes and studied the influence of cholesterol content on platelet arachidonic acid metabolism. These workers demonstrated that thrombin when added to cholesterol-enriched platelets (pre-labelled with [^{14}C] arachidonic acid) led to the release of

significantly more arachidonic acid from pre-labelled platelet phospholipids compared to cholesterol-depleted platelets. Subsequent conversion of arachidonic acid to thromboxane B_2 was also highly significantly enhanced in the cholesterol-rich platelets. Stuart *et al* (1980) postulated that the increased thrombin-stimulated release of arachidonic acid from platelet phospholipids could be due to previously described effects of cholesterol on membrane fluidity (Shattil and Cooper, 1978) and that changes in membrane fluidity might affect the activity of the phospholipase A_2 enzyme which is principally responsible for the release of arachidonic acid. Further findings in this study may have a direct bearing on the findings of increased malondialdehyde formation in hypercholesterolaemic platelets reported in this thesis. Stuart *et al* (1980) demonstrated both thromboxane B_2 and the hydroxy fatty acid 12 L-hydroxy-5,8,10,heptadecatrienoic acid (HHT) production from arachidonic acid was increased in the "cholesterol-rich" platelets. It is important to bear in mind that malondialdehyde formation is not just a function of the activity of the enzyme thromboxane synthetase. Indeed a major alternative pathway for the metabolism of the cyclic endoperoxides is by further degradation to HHT and malondialdehyde (Moncada and Vane, 1978). Therefore the increase in HHT and thromboxane B_2 formation suggests that enhanced conversion of arachidonic acid in "cholesterol-rich" platelets is probably due to enhanced activity of the cyclo-oxygenase enzyme since this enzyme rather than thromboxane synthetase which acts subsequently, is rate determining for the reaction (Stuart *et al*, 1980). In a recent study Tandon *et al* (1983) have examined the effect of cholesterol loading of platelets, leading to changes in membrane microviscosity, on thrombin receptors. They showed that the number of thrombin receptors increased with increasing membrane viscosity while binding affinity decreased. Receptor number appeared to be the principal determinant of platelet responsiveness, however coupling between

occupied receptor and bioresponse was unaffected by changes in membrane viscosity (Tandon *et al*, 1983).

Evidence of increased platelet malondialdehyde formation in hypercholesterolaemic individuals presented in this thesis gives support to an attractive hypothesis proposed by Fogelman *et al* (1980) which may have important consequences for platelet/lipid interactions. As discussed in the introduction to this thesis platelet-derived growth factor released from the alpha-granules of platelets may have marked effects on lipoprotein cell interactions. In addition malondialdehyde may also be an important platelet product capable of such effects. The foam cells so characteristic of atheroma may have their origin in macrophage-monocytes which have receptors for chemically altered LDL. Unlike normal LDL receptors they are not down regulated by increasing cellular cholesterol concentration (Goldstein *et al*, 1979). One type of chemically altered LDL taken up by macrophages resulting in massive cholesterol accumulation in these cells is malondialdehyde-modified LDL (Fogelman *et al*, 1980). The hypothesis would be that malondialdehyde, a stable end-product of the prostaglandin cascade released from platelets could lead to chemical modification of LDL rendering it recognisable by macrophage receptors leading to cholesterol accumulation in these cells.

Summary

The results reported in this thesis suggest that in diabetic and hyperlipidaemic subjects, two groups at risk from premature vascular disease, platelet function as assessed by measurement of the platelet-specific proteins (markers of *in vivo* platelet release reaction) is enhanced. In addition the activity of the prostaglandin pathway in response to stimulation with arachidonic acid is increased as evidenced by increased malondialdehyde formation. In addition, in diabetic subjects whole blood platelet aggregation is enhanced; platelet prostacyclin sensitivity is reduced and in diabetic animals aortic prostacyclin production is reduced. Overall these findings appear to be related to the altered metabolic state found in diabetes and hyperlipidaemia rather than to pre-existing vascular disease. These alterations in platelet function may contribute to the increased vascular risk seen in these subjects. However more work is needed to pin-point the exact pathophysiology of platelet abnormalities in these conditions particularly whether they are secondary to endothelial cell malfunction. In terms of therapeutic intervention the studies reported here argue for careful diabetic control not only in terms of glycaemic control but also for the achievement of normolipaemia. For the hyperlipidaemic subjects they provide a further justification for treatment of their high lipid levels. However it must be remembered that there are as yet no prospective epidemiological studies relating platelet function to the development of vascular disease as no satisfactory assessment of platelet function is available for such studies. In addition studies of a prospective nature which have intervened with platelet-active drugs throw no light on the 'platelet hypothesis' as as yet there is no satisfactory drug available.

Until there are more satisfactory measures of overall platelet function and effective therapeutic interventions which can be put to the test in carefully controlled prospective studies then the conclusions of the work presented in this thesis and other studies conducted along similar lines must remain tentative.

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